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# FACTORS AFFECTING COLORIMETRIC URINARY 17-KETOSTEROID DETERMINATIONS[1]

IRA T. NATHANSON AND HILDEGARD WILSON

*From the John Collins Warren Laboratories of the Cancer Commission of Harvard University, Massachusetts General Hospital*

BOSTON, MASSACHUSETTS

THERE are a number of procedures for estimating the 17-ketosteroids in urine extracts by the use of m-dinitrobenzene in alkaline alcoholic solution. These various methods do not give similar results with the same urine extract (Friedgood and Berman, 1941; Strickler, *et al.* 1941). In this laboratory normal values for 17-ketosteroid excretion obtained with a photoelectric modification of the method of Oesting (1937) have been higher than those reported with some other procedures. The present study was undertaken to establish the usual magnitude of such discrepancies and the basis for these differences.[2]

The following methods, which represent those most commonly used for the assay of 17-ketosteroids, were selected for study. All utilize the photoelectric colorimeter. A. The Callow (1938) procedure as adapted by Langstroth and Talbot (1939a) and Talbot, *et al.* (1940a). B. The procedure of Holtorff and Koch (1940). C. The procedure of Friedgood and Whidden (1939). D. A photoelectric modification of the method of Oesting (1937). For the details of each method see table 1. Each procedure will hereafter be referred to by the letters A, B, C or D. In the order named they employ a decreasing concentration of reagents or of alcohol in the reaction mixture. For this reason the reaction mixtures of A and B are termed 'concentrated,' while those of C and D are termed 'dilute.'

An extract of pooled urine was first assayed by these 4 methods to establish the variations resulting from use of the different procedures. The directions for the various methods were followed exactly. In

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[2] We are indebted to Dr. E. Oppenheimer of Ciba Pharmaceutical Products, Inc., Summit, N. J., and Dr. E. Schwenk of the Schering Corporation, Bloomfield, N. J., for samples of androsterone and dehydroisoandrosterone and to Dr. S. Lieberman of the Department of Organic Chemistry of Harvard University for the etiocholanone used in these experiments.



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TABLE 1. DETAILS OF THE FOUR PROCEDURES STUDIED, K VALUES AND ERRORS IN ANDROSTERONE CALIBRATION CURVES, AND OBSERVED KETOSTEROID CONTENT OF THE POOLED EXTRACT EXPRESSED AS ANDROSTERONE EQUIVALENT

| Procedure | | A | B | C | D | |
|---|---|---|---|---|---|---|
| Author | | Talbot, *et al.* (1939, 1940) | Holtorff and Koch (1940) | Friedgood and Whidden (1939) | After Oesting (1937) | |
| Reaction Mixture | Androsterone or extract | 0.2 cc. in 100% alcohol | 0.2 cc. in 95% alcohol | 0.2 cc. in 95% alcohol | 10 cc. in 60% alcohol | |
| | m-Dinitrobenzene | 0.2 cc. 1.16% in 100% alcohol | 0.2 cc. 2% in 95% alcohol | 0.2 cc. 2% in 100% alcohol | 2 cc. 2% in 95% alcohol | |
| | Potassium hydroxide | 0.2 cc. 2.5 N in 100% alcohol | 0.2 cc. 5 N aqueous | 0.2 cc. 2 N aqueous | 2 cc. 15% aqueous | |
| Alcohol concentration in reaction mixture, % | | 100 | 63 | 64 | 51 | |
| Potassium hydroxide concentration in reaction mixture, N | | 0.83 | 1.67 | 0.67 | 0.38 | |
| Time of color development, min. | | 80 | 45 | 75 | 60 | 90 |
| Dilution before reading | | 20 cc. 95% alc. | 10 cc. 95% alc. | 10 cc. 95% alc. | None | None |
| Androsterone Calibration Curves | Number of observations— | 41 | 40 | 31 | 20 | |
| | Mean K value[1] | 3.90 | 6.22 | 2.85 | 0.194 | |
| | Standard deviation (mean error of a single observation) | 0.302 | 0.287 | 0.140 | 0.0041 | |
| | Coefficient of variation (% error of a single observation) | 7.8 | 4.6 | 4.9 | 2.1 | |
| Urine Extract Assays | Number of observations | 22 | 43 | 52 | 9 | 7 |
| | Average mg. androsterone equivalent found per cc. pooled extract[2] | 0.238 | 0.327 | 0.350 | 0.821[3] | 0.689[3] |

[1] K = L/mg. androsterone used, where L is 2 minus the logarithm of the galvanometer reading.
[2] Various amounts of the pooled extract were used, and the results calculated for content per cc. and averaged.
[3] Result corrected for original pigment color of the extract.

further studies the time of color development alone was varied in order to ascertain the possible rôle of this factor in producing the discrepancies observed with the different procedures.

## METHODS

*Preparation of the neutral fraction of pooled urine.* Eleven liters of pooled urine from healthy adult males were hydrolyzed by boiling with hydrochloric acid and extracted with benzene after the method of Smith and Smith (1935). The 17-ketosteroids were separated from the estrogens using a procedure essentially that of Gallagher, *et al.* (1937). The ether solution (neutral fraction) containing the 17-ketosteroids was evaporated to dryness and the residue dissolved in 500 cc. of 95 per cent alcohol. This solution constituted the pooled extract. Extracts of 24-hour collections of urine from 26 individuals were similarly prepared for a comparison of results with those from the pooled extract.

The reagents used were U. S. Industrial alcohol, Eastman No. 99 *m*-dinitrobenzene purified according to Callow, *et al.* (1938) and Merck reagent

potassium hydroxide except for procedure B in which the Eimer and Amend electrolytic potassium hydroxide specified by the authors was substituted.

*Colorimetric procedure.* Reaction tubes were placed in a constant temperature water bath at 25±0.2° C. in semi-darkness. Readings were made with an Evelyn photoelectric colorimeter using a Rubicon No. 520 filter. Concentrations of androsterone and of the urine extract were selected so that readings would fall between 30 and 80 on the galvanometer scale thus insuring greater accuracy.

Crystalline androsterone was used as a reference standard, hence the observed 17-ketosteroid content of the urine extract is expressed as mg. androsterone equivalent. Calibration curves were made with graded concentrations of androsterone. The proportionality constant K was calculated from K = L/mg. androsterone used, where *L* is 2 minus the logarithm of the galvanometer reading.

*Correction for absorption due to pigment originally present in the neutral fraction of urine extract.* With procedures A, B and C the absorption due to pigment was too small to influence the results in the present study, and a correction was unnecessary. With procedure D pigment color formed a far larger proportion of the total color after reaction, hence corrections according to Holtorff and Koch (1940) have been applied to all of the data on the pooled extract obtained with procedure D.

## EXPERIMENTS AND RESULTS

*Experimental error.* To test the reproducibility of calibrations on different days and with different batches of reagents, numerous curves were prepared based on determinations made throughout the year. The variations in K values in those calibrations made at the reading times recommended by the various authors is shown in table 1. Substantially the same magnitude of deviation was found at other reading times. Results obtained by procedure A were less reproducible than the others. As shown in table 1, single observations were in error by an average of ±7.8 per cent. The sensitivity and rapidity of the reaction in absolute alcohol probably allows the final color in both reagent blanks and tubes containing ketosteroids to be easily influenced. Thus several observations were required to reduce the error of a result.

*Relation between concentration and reading. Adherence to Beer's law.* The amounts of crystalline androsterone used gave a linear relationship between reading and concentration with procedures A, B and C. Variations in K were irregular due to errors inherent in each method rather than to deviation from Beer's law. With procedure D a slight deviation from Beer's law was found when the period of color development was lengthened.

In the case of the urine extract, variations in observed androsterone equivalent with increasing volume are shown in table 2. The values with procedure A were constant within experimental error over a four-fold range of extract concentration. The same was true of method B for amounts which gave readings above 30 on the galvanometer. Possible errors from this source may be minimized in

TABLE 2. EFFECT OF CONCENTRATION OF THE POOLED EXTRACT ON THE OBSERVED ANDROSTERONE EQUIVALENT

| | cc. Extract[1] | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0.05 | 0.10 | 0.15 | 0.20 | 0.30 | 0.40 | 0.50 | 0.60 | 0.80 | 1.0 | 2.0 |
| | *Procedure A, read at 80 minutes* | | | | | | | | | | |
| Number of observations | | | | 5 | | 5 | | 3 | 1 | | |
| Androsterone equivalent mg./cc. | | | | 0.250 | | 0.249 | | 0.246 | 0.240 | | |
| | *Procedure B, read at 45 minutes* | | | | | | | | | | |
| Number of observations | 6 | 4 | 4 | 5 | | 2 | | | | | |
| Androsterone equivalent mg./cc. | 0.328 | 0.334 | 0.336 | 0.338 | | 0.313 | | | | | |
| | *Procedure C, read at 75 minutes* | | | | | | | | | | |
| Number of observations | | 3 | | 4 | 3 | 4 | 3 | 3 | 3 | 3 | |
| Androsterone equivalent mg./cc. | | 0.349 | | 0.348 | 0.345 | 0.343 | 0.325 | 0.310 | 0.300 | 0.289 | |
| | *Procedure D, read at 60 minutes* | | | | | | | | | | |
| Number of observations | | | | | | 3 | | | | 4 | 2 |
| Androsterone equivalent mg./cc.[2] | | | | | | 0.926 | | | | 0.790 | 0.722 |

[1] Amounts over 0.2. cc. had been concentrated to 0.2 cc. or less with procedures A, B and C.
[2] Corrected for original color of the extract.

routine assays by adjusting samples to read between 50 and 60. With procedures C and D there was a marked drop in the values as the amount of extract was increased. It has been frequently observed in this laboratory that the deviation from Beer's law observed with procedure D was eliminated when extracts were decolorized with norite.

*17-Ketosteroid content of the pooled extract as determined by various procedures.* Assay of the pooled extract by the 4 different procedures revealed marked variations in the observed 17-ketosteroid content per cc. (table 1). In general the values were higher when the reagent or alcohol concentration was lower. The values with methods B, C and D were about 1.4, 1.5, and 3 times that with A, respectively. These findings show the approximate variation to be expected with these different procedures.

When samples of the pooled extract were treated with the minimum

amount of norite for complete decolorization the mean of 4 assays with method D was about 68 per cent of the values obtained without norite. Thus results in laboratories using procedures similar to D on charcoal-treated extracts are more likely to be approximately 1.5 to 2 rather than 3 times greater than the results when procedures A, B or C are used without charcoal. Such ratios were also found in the present work on various other extracts. Other workers have shown (Talbot and Langstroth, 1939; Pincus and Pearlman, 1941), and it has been partially confirmed in this laboratory, that treatment with charcoal removes not only pigment color but also chromogenic substances which may include some ketosteroids.

*Rate and extent of color development of androsterone and extract (fig. 1).* It can be seen that as the reaction progresses there is a gradual increase in the intensity of the color until it becomes constant. Preliminary observations had shown that the rate of color change of androsterone was different with each of the 4 procedures. Hence it was obvious that similar studies on the pooled urine extract were desirable. Variations in rate of change of color intensity made it seem likely that the discrepancies among the 4 procedures could be partially if not completely explained on this basis.

As stated, graded concentrations of androsterone and of the pooled extract were chosen for each procedure so that galvanometer readings would fall in the optimum range. The change in color intensity was then determined at various time intervals. The results showed that increased concentration of alcohol or of potassium hydroxide, or a greater total concentration of the reaction mixture effected a more rapid attainment of a more intense color per unit weight of reactant (fig. 1). The observed L values with procedure A are relatively lowered because of the greater dilution before reading. Similar observations have been made by Zimmermann (1936) and Friedgood and Whidden (1940).

With all procedures the extract developed color at a faster initial rate than androsterone and reached a maximum sooner. Concentrated reaction mixtures tended to equalize the rates of color development of standard and urinary steroid mixtures, while in dilute reaction mixtures the extract reached maximum color much sooner than androsterone. Thus the difference in reaction time between androsterone and urine extract was least with procedure A and greatest with D.

*Effect of reading time on the observed 17-ketosteroid content of the pooled extract. Optimum reading time.* Since the extract invariably developed a greater proportion of its maximum color than androsterone during the initial stages of the reaction, it was to be expected that high 17-ketosteroid values would be obtained after short reaction periods. Thus when samples of the pooled extract were compared with androsterone standards after various periods of color development the observed androsterone equivalent of the extract was always

highest at the earliest reading time (table 3). In the later stages of the reaction, when both standard and extract approached maximum color, the 17-ketosteroid values progressively decreased, and tended to become constant with each procedure. Values obtained by the 4 methods were in much closer agreement at or near the androsterone

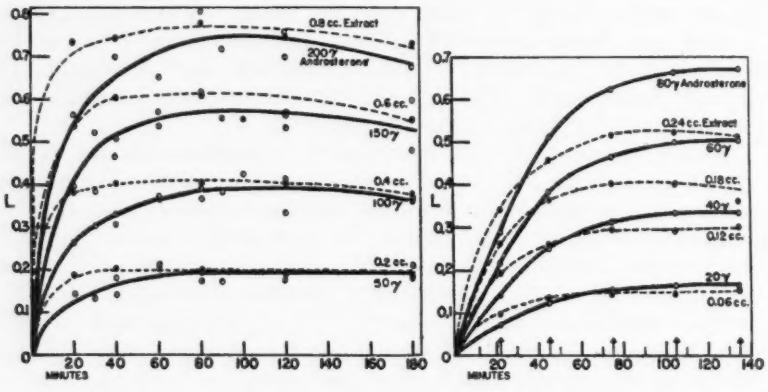


FIG. 1. Color-time curves of androsterone and the pooled extract compared in the four procedures. Solid lines are androsterone, broken lines are the extract. A. (*left*) Results with the procedure of Talbot, *et al.* (absolute alcohol). Each point represents a single observation. B. (*right*) Results with the procedure of Holtorff and Koch (5 N aqueous potassium hydroxide). Each point is an average of 3 or more observations.

maxima than at previous times. These lower values are probably more accurate, since standard and extract are as close as possible to the same extent of reaction when both have reached constant or maximum color. Thus this stage represents the optimum reading time.

On the basis of these observations (table 3) the optimum reading time with procedure A is about 80 minutes and with procedure B about 105 minutes. Androsterone reacted much more slowly than the extract with procedures C and D, and the extract tended to fade before maximum androsterone color was reached.[3] Longer reading times than those originally specified can nevertheless be expected to give more accurate values.

*Optimum reaction mixture. Choice of procedure.* It is clear that the optimum reaction mixture for obtaining quantitative values is that which brings the various urinary 17-ketosteroids to most nearly the same extent of reaction at the optimum reading time. Observations

[3] The term 'fade' denotes a reaction mixture which had less color than another of the same composition read at an earlier time. It does not denote the loss of color which occurred 10 to 15 minutes after reaction mixtures had been diluted with alcohol in procedures A, B and C. The pooled extract had not faded at 5 hours with procedure D, but beyond this time fading occurred with several of the other extracts to be described.

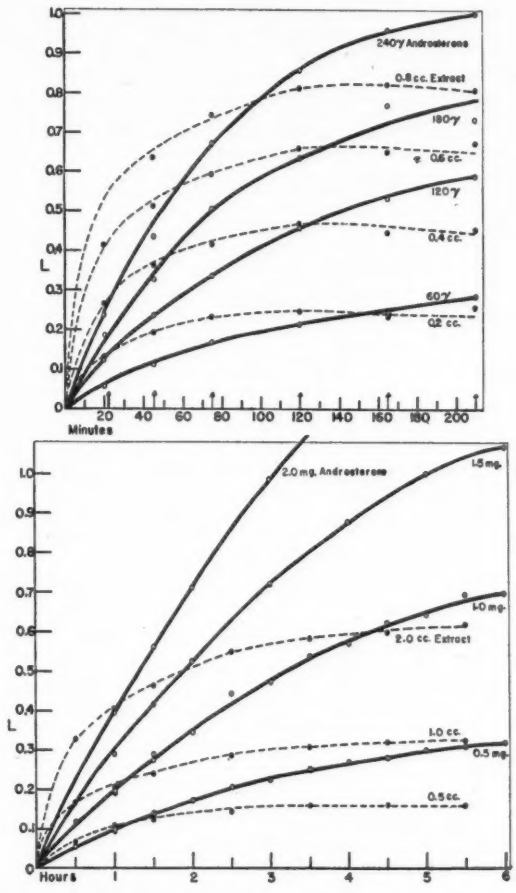


Fig. 1, *con't.* C. (*above*) Results with the procedure of Friedgood and Whidden (2 N aqueous potassium hydroxide). Each point is an average of 3 or more observations. D. (*below*) Results with the modified Oesting procedure (15% aqueous potassium hydroxide and 60% alcohol). Each point represents a single observation.

on crystalline preparations of several of the known urinary 17-ketosteroids indicate that reaction mixtures employing a high concentration of either alcohol or potassium hydroxide are best in this respect. The relative behavior of androsterone and dehydroisoandrosterone may be cited as an example.

With procedure A, the K value for androsterone was approximately 93 per cent of that for dehydroisoandrosterone at 40 minutes, while at 80 minutes, as has also been shown by Langstroth and Talbot (1939b), these gave substantially the same color intensity. The cor-

TABLE 3. EFFECT OF READING TIME ON THE OBSERVED 17-KETOSTEROID CONTENT OF THE POOLED URINE EXTRACT, EXPRESSED AS ANDROSTERONE EQUIVALENT

| | Reading Time | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 20 min. | 45 min. | 60 min. | 75 min. | 105 min. | 2 hr. | 2.5 hr. | 4.5 hr. | 5.5 hr. |
| | *Procedure A* | | | | | | | | |
| Number of observations | 4 | 6 | 7 | 6 | | 8 | | | |
| Androsterone equivalent mg./cc. | 0.325 | 0.273 | 0.255 | 0.255 | | 0.240 | | | |
| | *Procedure B* | | | | | | | | |
| Number of observations | 10 | 12 | | 19 | 19 | | 11 | | |
| Androsterone equivalent mg./cc. | 0.422 | 0.322 | | 0.298 | 0.275 | | 0.277 | | |
| | *Procedure C* | | | | | | | | |
| Number of observations | 4 | 6 | | 12 | | 12 | | | |
| Androsterone equivalent mg./cc. | 0.670 | 0.478 | | 0.361 | | 0.318[1] | | | |
| | *Procedure D* | | | | | | | | |
| Number of observations | | | 3 | | 3 | | 3 | 3 | 3 |
| Androsterone equivalent mg./cc.[2] | | | 0.810 | | 0.655 | | 0.565 | 0.460 | 0.455 |

[1] Extract had faded at next interval, 2.75 hours.
[2] Corrected for original color of the extract.

responding percentages with procedure B were 86 at 45 minutes and 95 at 105 minutes. With procedure D however, the androsterone K was only 68 per cent of that for dehydroisoandrosterone at 1 hour, and only rose to 72 per cent at 6 hours when both reactions were essentially at a maximum. Further unpublished observations show that if either the alcohol or potassium hydroxide concentration of method D is increased these 17-ketosteroids will react to more nearly the same extent. Thus the relative extent of reaction of various 17-ketosteroids is dependent on either the alcohol or potassium hydroxide concentration, or both. The K value for 3$\alpha$-hydroxy-etiocholanone-17 was higher than for dehydroisoandrosterone at all times with procedures A and B, although closest at the time of maximum color, showing that all 17-ketosteroids do not give the same maximum color.

These figures illustrate why extension of the reading time for procedure B gave assay values in good agreement with those obtained

by procedure A, while values obtained with D were considerably higher even when the reading time was prolonged to 6 hours. It appears that dilution of the reaction mixture decreases the maximum extent of reaction of androsterone more than of certain other urinary 17-ketosteroids, leading to higher and inaccurate values no matter how the reading time is adjusted.

Polarographic determinations, which measure only 17-ketoster-

TABLE 4. RECOVERY OF ADDED ANDROSTERONE. RECOVERY IS THE DIFFERENCE IN OBSERVED ANDROSTERONE EQUIVALENT BETWEEN THE ORIGINAL EXTRACT AND THE EXTRACT PLUS ANDROSTERONE

| Reading Time | 22 min. | 45 min. | 75 min. | 2 hr. | 4 hr. |
|---|---|---|---|---|---|
| | *Procedure A* | | | | |
| Number of observations | | 4 | 4 | 4 | |
| Mg. per cc. | | 0.21 | 0.21 | 0.25 | |
| Percentage recovery | | 84 | 84 | 100 | |
| | *Procedure B* | | | | |
| Number of observations | 7 | 7 | 7 | | |
| Mg. per cc. | 0.26 | 0.23 | 0.25 | | |
| Percentage recovery | 104 | 92 | 100 | | |
| | *Procedure C* | | | | |
| Number of observations | | 9 | 6 | 9 | |
| Mg. per cc. | | 0.25 | 0.26 | 0.24 | |
| Percentage recovery | | 100 | 104 | 96 | |
| | *Procedure D* | | | | |
| Number of observations | | | 2 | 2 | 2 |
| Mg. per cc. | | | 0.27 | 0.25 | 0.23 |
| Percentage recovery | | | 108 | 100 | 92 |

0.25 mg. androsterone was added per cc. extract.
Results are averaged from two extract concentrations.

oids, support the contention that the lower values are the most accurate. Using the polarograph similar to that developed by Wolfe, Hershberg and Fieser (1940), the pooled extract was found to contain 0.195 mg. of 17-ketosteroids per cc. This is lower than the androsterone equivalent found by any of the colorimetric procedures, but nearest to the values obtained with procedures A or B (table 2).[4]

*Recovery of added androsterone.* Crystalline androsterone was added to an aliquot of the pooled extract and the resulting total androsterone

[4] We are indebted to Dr. N. T. Werthessen of the Beth Israel Hospital, Boston, for polarographic measurements.

equivalent determined simultaneously with that of samples of the original extract. Androsterone recovery remained essentially quantitative with the different procedures at various reading times (table 4). The irregular variations are probably due to the fact that a determination by difference doubles the experimental error.

In another experiment, using only procedure B, five graded amounts of androsterone were added to 0.1 cc. samples of the pooled

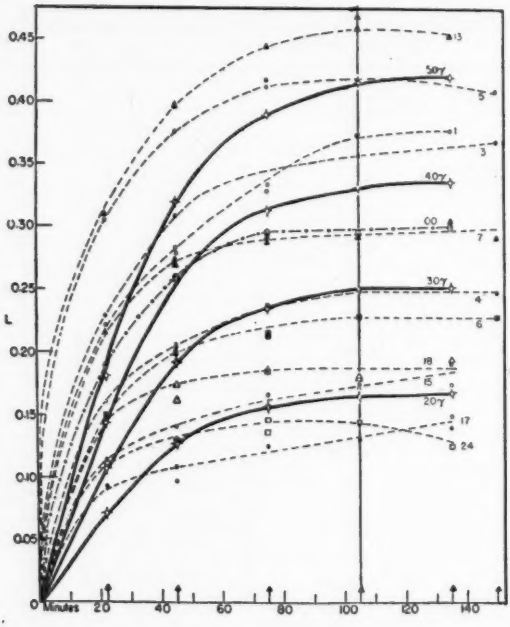


FIG. 2. Color-time curves on various urine extracts with the procedure of Holtorff and Koch (B) showing attainment of maximum color at 105 minutes (1¾ hours) by most extracts and by androsterone. Broken lines are urine extracts from individuals except for No. 00 which is the pooled extract. Solid lines are androsterone curves calculated from average K values at each time of observation.

extract, and readings taken at 105 minutes. At each androsterone concentration 94 to 100 per cent was recovered by difference. Hence at this time K for androsterone was essentially the same in the extract as in pure solution. It is evident, therefore, that androsterone color development proceeded independently of other substances in the extract, or at least was not sufficiently altered to account for the more rapid reaction of urine extract.

### VARIOUS EXTRACTS

Since the above observations applied only to one pooled extract, it was desirable to check the findings on other extracts. Therefore

38 urine specimens from 26 individuals were chosen at random and studied. The subjects were of both sexes, including extremes of age, normal persons and those with various endocrine abnormalities.

It was important to ascertain that other extracts resembled the pooled extract in showing *a*) a time of maximum color attainment earlier or no later than that of androsterone, *b*) a lower 17-ketosteroid

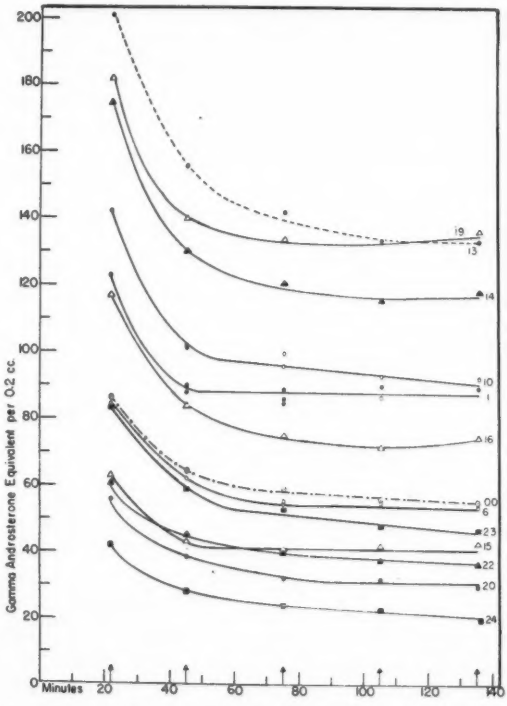


FIG. 3. Observed androsterone equivalent of various extracts with the procedure of Holtorff and Koch (B) plotted against reading time. These curves show that results tend towards a constant minimum near 105 min. (1¾ hours). The ordinate is gamma of androsterone equivalent per 0.2 cc. extract except for curve No. 13 which represents 0.1 cc., and No. 00 which represents 0.18 cc. of the pooled extract. These curves are in the order of total 24-hour urinary 17-ketosteroid content, since all extracts had been diluted to the same original volume.

content at the time of maximum androsterone color than at previous times, *c*) agreement between the 17-ketosteroid values obtained with procedure A at 80 minutes and procedure B at 105 minutes, and *d*) higher values with procedure D even at long reading times. These assumptions were all substantiated.

The rates of color development of the various extracts were studied using procedure B. As with the pooled extract, the initial color development was faster than with androsterone (fig. 2). Almost

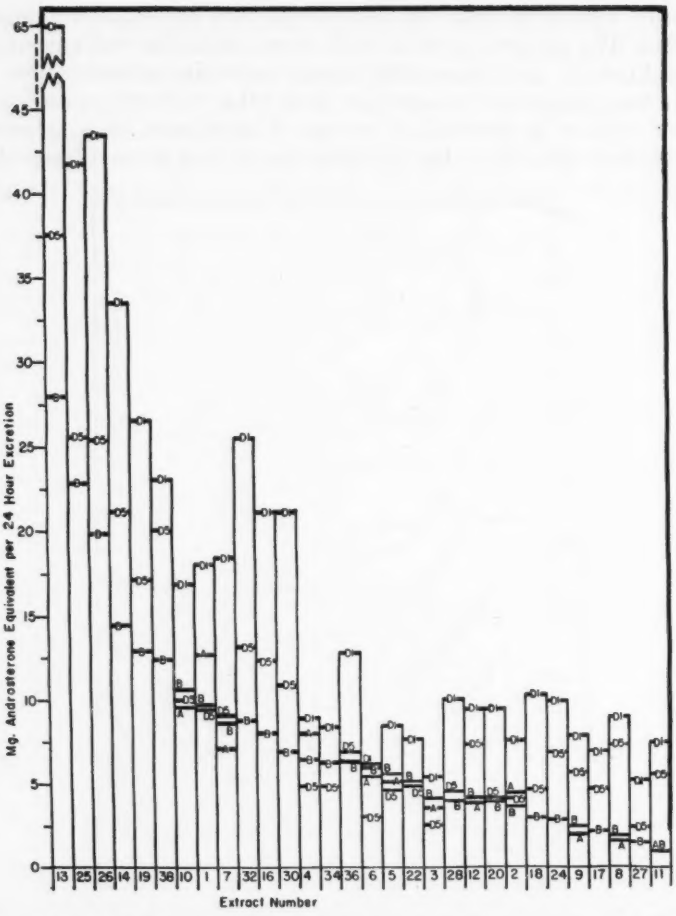


FIG. 4. Observed androsterone equivalent in 30 individual urine extracts by different procedures. Each column represents one extract; crossbars show the various values. D1 and D5 indicate values by the Oesting modification (D) when readings were taken at 1 and 5 hours, respectively. B indicates values by the procedure of Holtorff and Koch when readings were taken at 1¾ hours. A indicates values by the procedure of Talbot, *et al.*, on extract numbers 1–12. Extracts are arranged from left to right in the order of decreasing values by procedure B.

all extracts had attained a color maximum at 105 minutes, while the observed 17-ketosteroid content reached a constant minimum at this time (fig. 3). Thus the optimum reading time of 105 minutes for procedure B was confirmed.

Twelve extracts assayed by procedure A gave values in close agreement with those found at 105 minutes with procedure B (fig. 4).

*Comparison of 17-ketosteroid values obtained with different procedures.* The mg. androsterone equivalent excreted per 24 hours as

measured by different methods in most of these extracts are shown in figure 4. The aliquots used for procedure D were decolorized with norite A.[5] Those for methods A and B were not decolorized, but a correction for original pigment was applied. When the dilute reaction mixtures of procedure D were read at 1 hour all values were higher than with B and most were much higher. At 5 hours, when maximum androsterone color is closely approached with procedure D, all values were in better agreement with B, some were almost identical, and some even lower.

The fact that some values obtained with method D at 5 hours were below those obtained with B appears to be due to the effect of norite. Aliquots of 26 of these same extracts which had not been treated with norite showed values at 5 hours well above those with methods A and B.

The general trend from high to low values for 17-ketosteroid excretion is the same for these different procedures (fig. 4). Thus values in one laboratory may be expected to bear the same relation to an established normal range as in another using a different procedure. However, the low values obtained with the absolute alcohol or 5 N potassium hydroxide procedures (A and B) are unquestionably much closer to the true 17-ketosteroid content of urine than the high values found with the 60 per cent alcohol method, D.

## DISCUSSION

It is evident that none of these procedures is ideal for the assay of an extract containing a complex mixture of 17-ketosteroids. Errors are partly due to the inadequacy of androsterone as a reference standard, since this 17-ketosteroid reacts less like the urine extracts than others. Of the 17-ketosteroids studied, androsterone developed the least color per unit weight, and required the longest time to reach maximum color. Standards containing both androsterone and dehydroisoandrosterone have been suggested (Friedgood and Whidden, 1941), but since the latter hormone normally comprises only 5 to 10 per cent of the total urinary androgens (Baumann and Metzger, 1940), their use provides no great improvement. The characteristic color-time curve of the neutral urinary steroid mixture is unlike that for either of these hormones. Our observations suggest that 3$\alpha$-hydroxy-etiocholanone-17 behaves more like the extracts and is therefore a more suitable reference standard. An ideal standard would be one containing a mixture of the various known 17-ketosteroids in the proportion found in the urine of normal people. The difficulty of establishing a standard lies in the fact that pathological urines contain other 17-ketosteroids and frequently in different proportions. Hence it is more practical at present to select a single reference standard which most closely resembles urine extracts.

[5] Norite was used in order to make results comparable to those routinely obtained in this and other laboratories using a similar procedure.

In addition, the time of reaction and the concentration of reagents should be such that a reference standard and the various other urinary 17-ketosteroids will give equivalent color intensities. This condition is best fulfilled in concentrated reaction mixtures with an adequate period of color development. Procedure A employs the highest percentage of alcohol, but in procedure B concentrations of potassium hydroxide and m-dinitrobenzene are higher. Extending the reading time of procedure B to 105 minutes gives essentially the same values as with A, and the inconvenience and time required for frequent preparation of alcoholic potassium hydroxide in procedure A is avoided, as is the necessity for anydrous conditions in reagents and extracts. In the present work more reproducible results were obtained with procedure B. For routine examination of urine extracts we therefore prefer the procedure of Holtorff and Koch, B, but with a reading time of 105 minutes.

It is to be emphasized that the color produced by m-dinitrobenzene in the neutral fraction is not necessarily due to 17-ketosteroids only, but to any chromogenic substances which may be present. Further purification such as that proposed by Baumann and Metzger (1940), or further fractionation into ketonic and non-ketonic, alcoholic and non-alcoholic steroids (Talbot, *et al.* 1940b; Pincus and Pearlman, 1941) may make more accurate results possible. The correction formula of Fraser, *et al.* (1941), based on a formula of Gibson and Evelyn (1938), has been devised to subtract interfering color due to non-ketonic substances in an unpurified neutral fraction when proper controls are used (Talbot, *et al.*, 1942). Experiments on these points are in progress.

## SUMMARY

Four established procedures for the colorimetric estimation of 17-ketosteroids by the m-dinitrobenzene reaction have been applied to the same neutral fraction of a pooled urine extract. These differ in the concentration of reagents or of alcohol employed in the reaction mixtures. Each procedure gave a different value for the 17-ketosteroid content of this urine extract. The lowest values were found with methods employing either a high percentage of alcohol or concentrated potassium hydroxide, or both.

The time course of color development of a crystalline androsterone reference standard and of the urine extract has been followed with the four procedures. In each case the extract reacted at a faster initial rate and subsequently reached constant color intensity. In concentrated reaction mixtures androsterone color also became constant at a somewhat later time. Therefore readings made before this plateau was reached gave higher values for 17-ketosteroids. This partially explains the discrepancies between values reported by the various methods, since rates of reaction are different with each. Adjustment

of the reading times resulted in closer agreement between the various methods.

In concentrated reaction mixtures only, three crystalline 17-ketosteroids studied reached nearly equivalent color intensities with adequate reaction times. This suggests that the lower values obtained under such conditions are more accurate than those in dilute reaction mixtures. Comparable values were obtained in concentrated reaction mixtures with the absolute alcohol procedure (after Callow) and with the procedure employing 5N aqueous potassium hydroxide (Holtorff and Koch), provided an adequate period of color development was allowed for the latter. The modified Holtorff and Koch procedure is preferred as it involves less technical difficulty and a smaller error.

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# INACTIVATION OF ESTRONE AND DIETHYLSTILBESTROL BY MICROORGANISMS[1,2]

BERNHARD ZONDEK AND FELIX SULMAN
*From the Hormone Research Laboratory of the Hebrew University*
JERUSALEM, PALESTINE

ESTRONE is inactivated in the liver and spleen of warm-blooded animals (Zondek, 1934a, b; 1935; 1941; Zondek and Sklow, 1941), in the organism of cold-blooded animals (Zondek, 1934a, b; Engel and Navratil, 1937), and in plants (von Euler and Zondek, 1934).

The view put forward by Zondek that an enzyme is responsible for the process of inactivation has been supported by the work of Westerfeld (1940). This author obtained from mushrooms a tyrosinase extract which converts tyrosine into melanin and decomposes estrone. Unpublished investigations by Zondek and Sklow have shown that hyacinth roots and cauliflower juice decompose estrone although they contain no melanin-forming tyrosinase. It was found, furthermore, that melanin-forming tyrosinase prepared from mealworm larvae does not inactivate estrone. On the other hand, beet root and potato juice contain tyrosinase, and, like mushroom extracts, these decompose estrone. As will be shown below, Proteus X Kingsbury and a certain strain of B. mesentericus decompose estrone but fail to form melanin from tyrosine.

It is well known that estrone resists putrefactive decomposition in natural conditions. In the laboratory, however, we have regularly observed that aqueous solutions of estrone kept for two months without sterile precautions at room temperature undergo a considerable loss of potency, and that aqueous solutions of diethylstilbestrol kept in like conditions deteriorate entirely, becoming yellowish in color, within about two weeks. Even oily solutions of estrogens and estrogen esters show a considerable loss of potency (90 per cent) if kept without sterile precautions at room temperature for more than a year. There appeared to be the possibility that microörganisms which decompose estrone and diethylstilbestrol might be widespread. It should be borne in mind that in general the influence of microörganisms on estrogens may be complicated in view of the finding of von Euler and Zondek (1934) that contact with top yeast increases the potency of estrone solutions.

For these reasons it seemed of interest to study the decomposition

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[1] Aided by a grant of the Ella Sachs Plotz Foundation.
[2] For technical reasons the publication of this paper was delayed for 2 years.

of estrone and diethylstilbestrol by various microörganisms. A collection of strains supplied by the Department of Hygiene and Bacteriology of the Hebrew University[3] was used.

## TECHNIQUE

The strains of bacteria and fungi were cultivated in Erlenmeyer flasks on 10 cc. of agar which contained 0.5 per cent marmite or 0.3 per cent bovril and 0.5 per cent peptone. After 48 hours approximately 10 billion ($10^{10}$) bacteria were found. To each such culture 0.2 $\mu$g. of estrone or diethylstilbestrol[4] of 1 cc. of an aqueous solution were then added.

Estrogens were prepared in aqueous solution as follows: 10 mg. of estrogen was dissolved in 5 cc. of 96 per cent alcohol. One-tenth cc. of the alcoholic solution was mixed with 50 cc. of distilled water. The alcohol was then evaporated *in vacuo*, the volume adjusted to 50 cc., and the solution autoclaved. One cc. of this solution contained 4 $\mu$g. (40 I.U. of estrogen or 20 M.U.). Sterile aqueous estrone solutions preserve their titer fairly well if kept in the refrigerator; diethylstilbestrol solutions deteriorate even in the refrigerator when kept for longer than 4 weeks.

The bacteria were tested as to activity on aqueous solutions of estrogen since we found that solutions in oil were relatively insusceptible to rapid bacterial action. Aqueous estrogen solutions were allowed contact with the bacteria for 48 hours at room temperature, and then for 24 hours at 37° C., and were then extracted and prepared for the Allen-Doisy test in the following manner: The agar was twice extracted with boiling alcohol, then twice extracted for two hours with boiling acetone and finally once with ether. These 5 extracts were pooled and evaporated in a wide-necked bottle. The gummy residue was taken up in ether; olive oil was then added and the ether was driven off at 60° C. An insoluble fraction which separated under these conditions could be discarded since the oily solution obtained from a parallel control run on sterile agar always contained 100 per cent of the estrogen originally added to the agar. The oily extracts were diluted with olive oil if necessary and titrated by injecting varying amounts into 5 mature female spayed mice. In all, 128 groups of 5 mice each, *i.e.* 640 mice, were employed. Every assay was made twice. An agar blank was always run parallel with the bacterial assay. The hormone recovery from the former was designated 100 per cent, and the decrease or increase of titer induced by contact with the microörganisms was calculated in percentage on this basis.

## RESULTS

The first series of the experiments comprised a collection of 32 strains of non-pathogenic microörganisms: Azotobacter chroococcum, Pseudomonas aeruginosa, Pseudomonas fluorescens, Pseudomonas sp. I (bile salt-decomposing strain), Pseudomonas sp. II (bile salt-

[3] We are indebted to Prof. I. J. Kligler, Head of the Department of Hygiene and Bacteriology of the Hebrew University, who kindly placed his collection of bacteria at our disposal. The authors wish also to acknowledge the kind help of Dr. M. Aschner, Assistant in the Department of Hygiene.

[4] We are indebted to the Teva Middle East Pharmaceutical and Chemical Works Co. Ltd., Jerusalem, for a supply of diethylstilbestrol.

decomposing strain), Serratia marcescens, Escherichia coli, Escherichia sp. (non lactose fermenting), Proteus vulgaris, Proteus X 2, Proteus X 19, Proteus X Kingsbury, Proteus sp. (isolated from chicks), B. subtilis Cohn, B. mesentericus sp. (an antagonist of other aerobic bacilli), B. mycoides, B. panis, B. laterosporus, B. niger, Proactinomyces sp. 2 (bile salt-decomposing strain), Proactinomyces sp. 3 (bile salt-decomposing strain), Proactinomyces sp. 7 (bile salt-decomposing strain), Mycobacterium lacticola, Actinomyces sp. I, Actinomyces sp. II, Asperigillus glaucus, Saccharomyces cerevisiae (bottom yeast), Saccharomyces cerivisiae (top yeast), Willia anomala, Schizosaccharomyces pombe, Hansenia apiculata, Torula lactis.

Among the 32 non-pathogenic strains examined only two inactivated estrone, Proteus X Kingsbury and B. mesenterious sp. If 4 $\mu$g. of estrone was added to a culture of these strains containing 10 billion ($10^{10}$) bacteria, less than 15 per cent of the potency of the estrone solution could be recovered, more than 85 per cent of the estrone being inactivated.

It was found that not every Proteus strain inactivated estrone: four members of the Proteus group (Proteus vulgaris, Proteus X 2, Proteus X 19, Proteus sp. chicken) did not inactivate estrone. The estrone-inactivating Proteus X Kingsbury is a relative of Proteus X 19, characterized by specific antigenic properties. Our strain was equally effective in inactivating estrone with a strain kindly placed at our disposal by Dr. Stuart, Head of the Government Laboratories Jerusalem.

B. mesentericus sp. was isolated from soil by the Department of Hygiene and Bacteriology of the Hebrew University and it differs *inter alia* from the non-inactivating subtilis strains by its strong antagonistic effect upon other bacilli.

In a number of undesignated proteus or mesentericus cultures, Proteus X Kingsbury and B. mesentericus sp. were easily recognized by means of their estrone-inactivating property.

Quantitative studies revealed that Proteus X Kingsbury and B. mesentericus sp. inactivated more than 85 per cent of the 4 $\mu$g. of estrone added to a culture containing about 10 billion ($10^{10}$) bacteria. A similar result was obtained when 400 $\mu$g. of estrone was added to the cultures. Working with larger quantities of estrone on cultures of about 10 billion ($10^{10}$) bacteria we found that after 48 hours at room temperature followed by 48 hours at 37°C. Proteus X Kingsbury inactivated 800 $\mu$g. of estrone of 1.4 mg. added to the culture. There remained, therefore, 600 $\mu$g. of active estrogen, *i.e.* 60 per cent. B. mesentericus sp. was less active.

The two strains, Proteus X Kingsbury and B. mesentericus sp., which inactivated estrone were allowed contact with diethylstilbestrol solutions under similar conditions. Both strains inactivated diethylstilbestrol as well as estrone.

Four of the yeast strains increased the estrogenic activity to 300 per cent of the original value (from 4 to 12 μg.) *viz:* Saccharomyces cerevisiae (bottom yeast), Saccharomyces cerevisiae (top yeast), Willia anomala, Schizosaccharomyces pombe.

In a second series of experiments 29 pathogenic strains of bacteria were examined with reference to their effect on aqueous estrone solutions. The following 29 strains have been available:[5] Eberthella typhi O, Eberthella typhi H, Eberthella typhi H, Salmonella partyphi[901] A, Salmonella paratyphi[901] B, Salmonella paratyphi[441] C, Salmonella enteritidis Gaertner, Salmonella London type, Bacterium enteritidis Breslau, Shigella dysenteriae, Shigella paradysenteriae Flexner, Shigella paradysenteriae Flexner W, Shigella paradysenteriae Flexner WX, Shigella paradysenteriae Flexner X, Shigella paradysenteriae Flexner Y, Shigella paradysenteriae Flexner VZ, Shigella paradysenteriae Flexner Z, Shigella Sonnei, Shigella ambigua (Schmitz), Brucella melitensis, Brucella abortus, Haemophilus influenzae, Corynebacterium diphtheriae, Staphylococcus aureus, Staphylococcus albus, Diplococcus pneumoniae, Streptococcus pyogenes, Neisseria catarrhalis, Monilia albicans. None of these strains changed the titer of the estrone solutions.

TABLE 1. CHANGE OF TITER OF AN AQUEOUS SOLUTION OF ESTRONE BY THE ACTION OF BACTERIA AND FUNGI[1]

| Name of Strain | Titer per cent |
|---|---|
| Proteus X Kingsbury | <15 |
| B. mesentericus sp. (antagonist of other aerobic bacilli) | <15 |
| Saccharomyces cerevisiae (bottom yeast) | 300 |
| Saccharomyces cerevisiae (top yeast) | 300 |
| Willia anomala | 300 |
| Schizosaccharomyces pombe | 300 |

[1] Nomenclature according to D. H. Bergey's Manual of Determinative Bacteriology, Baillière, Tindall & Cox, London, 5th ed. 1939.

## DISCUSSION

As seen in table 1, the activity of an estrone solution can be tripled by contact with bottom yeast, top yeast, pombe yeast and Willia yeast. This phenomenon was first described for top yeast by von Euler and Zondek in 1934, and lately also by Wettstein (1939), who explains the increase in titer as due to the conversion of estrone into estradiol by the yeast.

In view of Westerfeld's assumption (1940) that tyrosinase is the estrone-inactivating enzyme it seemed of interest to ascertain whether our estrone-inactivating strains of organisms, Proteus X Kingsbury

[5] We are indebted to Dr. J. Gurevitz, Head of the Bacteriological Laboratory of the Rothschild Hadassah University Hospital who kindly placed these strains at our disposal. The authors wish also to acknowledge the kind help of Dr. Daniela Weber.

and B. mesentericus sp., produce tyrosinase. In order to test this point, 10 billion ($10^{10}$) bacteria were allowed to act upon 1 to 10 cc. of a solution of 50 mg. of tyrosine in 100 cc. of 0.04 per cent sodium carbonate at a pH of 7.5 for 24 hours at 37° C. The test, which was also made on agar medium as well as on 1 to 10 cc. of normal saline, failed to demonstrate the presence of melanin-forming tyrosinase. The question whether estrone is inactivated by an enzyme of the tyrosinase group of a type which does not give a positive melanin test with tyrosine is under further investigation.

The ability of Proteus X Kingsbury and B. mesentericus sp. to inactivate estrone may serve to differentiate these from related organisms. It is possible that similar properties are possessed also by certain other strains. A broader survey of pathogenic bacteria from this point of view might be of practical interest. The 29 strains examined by us did not yield any noteworthy result.

## SUMMARY

Different microörganisms have been examined as to their effect on aqueous estrone and diethylstilbestrol solutions. Thirty-two non-pathogenic and 29 pathogenic strains were tested.

Aqueous solutions of estrone and diethylstilbestrol were inactivated by Proteus X Kingsbury and by a randomly isolated strain of B. mesentericus sp. Ten billion ($10^{10}$) bacteria were able to inactivate about 0.8 mg. of estrone.

The titer of aqueous solutions of estrone was tripled by contact with bottom yeast, top yeast, Willia yeast and pombe yeast. None of the pathogenic strains changed the biological titer of estrone.

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# THE INTRASPLENIC INJECTION OF ESTROGENS AND THEIR ESTERS

ALBERT SEGALOFF[1]

*From the Department of Anatomy, Wayne University, College of Medicine*
DETROIT, MICHIGAN

EVIDENCE has been gradually accumulating from diverse lines of investigation which would indicate that the liver is the site of extensive estrogen inactivation. Several workers have demonstrated independently that of all animal tissues studied *in vitro* only liver slices and liver extracts are capable of completely inactivating estrogens (Zondek, 1941; Heller, 1940). Studies *in vivo*, comparing the activity of ovaries or pellets of estrogen implanted in the portal field with that of ovaries or pellets having venous drainage into the systemic circulation, have also demonstrated that the liver is a major site of estrogen inactivation (Golden and Severinghaus, 1938; Biskind and Mark, 1939). There is, in addition, indirect evidence in that hepatotoxic agents apparently increase the effectiveness of endogenous and exogenous estrogens (Talbot, 1939; Pincus and Martin, 1940).

On the other hand, Cantarow and his co-workers (1942 a, b) have presented evidence which they interpret as indicating that a major loss of estrogenic hormone occurs by biliary excretion. Indeed, they proposed an entero-hepatic circulation of estrogen. In view of these many considerations it was thought of value to report in some detail attempts to quantitate the rôle of the liver in the inactivation of estrogens. To this end we have employed the intrasplenic injection of estrogens, as set forth in a preliminary note (Segaloff and Nelson, 1941).

## MATERIAL AND METHODS

In order to take advantage of the enhanced solubility of estrogens in sesame oil containing 5 per cent benzyl alcohol we have employed it as a solvent for all solutions.[2]

Each of the spayed rats was primed with 50 micrograms of estrone in 0.1 cc. of peanut oil 14 days prior to the test. The animals were adult females and were used repeatedly except when perisplenic adhesions occurred. Assays were conducted by giving the required amount of hormone, as a single injection in 0.05 cc. of the solvent. To insure the greatest possible accuracy all

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[1] Ciba Fellow in Endocrinology.

[2] The solvent was chosen at the suggestion of Dr. C. R. Scholz of Ciba Pharmaceutical Products, Inc., who has also been kind enough to prepare the solutions for these experiments.

injections were made with a Luer tuberculin syringe of 0.25 cc. capacity. Vaginal smears were taken with a moistened cotton-tipped tooth pick, 48, 54, 60 and 72 hours after the injection. All smears were stained with hematoxylin and eosin. A smear which showed only cornified epithelial cells at one or more of these times was classified as representing positive vaginal estrus. The end point was taken as the amount of estrogen which produced estrus in 50 per cent of at least 10 animals under these conditions.

In the animals used for intrasplenic injections with the spleen *in situ*, the spleen was exposed through a midventral incision, delivered to the outside, the injection made, and the site of the entrance of the needle into the spleen tied with an encircling silk ligature before withdrawal of the needle. The instruments and operative field were bathed with Zephiran chloride (Alba) 1:1000 and precautions were taken to maintain a clean, although not sterile, technique.

Spayed female rats with subcutaneous spleens were prepared by a three-stage operation. *a*) The spleen was delivered through a left lateral subcostal incision and a piece 1.5 cm. long was cut from one edge. The cut edge was approximated to a cut of similar length in the serratus anterior muscle over the 7th and 8th ribs and anchored by 3 silk stitches. The lateral subcostal incision was closed with a single loose silk stitch which also was passed through the center of the splenic mesentery and the skin was closed with clips. *b*) Two to 6 weeks later the skin over the spleen was again opened, the hilus of the spleen ligated and cut, the left ovary removed, the subcostal muscle incision closed with a continuous silk suture and the skin closed with clips. *c*) Two to 4 weeks following the second stage the right ovary was removed through a postero-lateral lumbar incision and 3 weeks later the animals were primed and used for assay. In making an intrasplenic injection in such a preparation the spleen was located by palpation, exposed by a skin incision, and the tip dissected free. The site of entrance of the needle into the spleen was tied with an encircling silk ligature as previously mentioned, to prevent leakage of the oil, and the skin was closed with clips.

All substances studied, except estrone, estriol and equilin, were readily soluble so that there was no difficulty in obtaining maximal responses. In the case of estrone, estriol and equilin the study was limited by their relative insolubility. Their maximal solubilities in 0.05 cc. of 5 per cent benzyl alcohol in sesame oil are 140, 12.5 and 125 μg., respectively.

In evaluating the rôle of the liver in the inactivation of the various estrogens and their esters a comparison has been made of the assay values obtained for each substance by the three methods of administration. This has been done on a basis of the ratios between the quantities of a given hormone required to produce vaginal estrus in 50 per cent of the test animals. All ratios will be given in the following sequence: subcutaneous, intrasplenic spleen subcutaneous, intrasplenic, spleen *in situ*, with the 50 per cent level of estrous response to injection in the subcutaneous spleen as unity. The use of these ratios permits a more ready evaluation of the effect of the liver upon the various estrogens. This procedure appears to be of particular value in taking into account the wide variations in apparent activity at the end organ which are shown by the different estrogenic substances.

## RESULTS

The results of the various experiments are tabulated in table 1. Each figure represents the injection of not less than 10 nor more than 25 animals. The three median figures, *i.e.*, the 50 per cent level and the levels immediately above and below that figure, for each route of

TABLE 1. RESULTS OF ESTROGEN INJECTION BY VARIOUS ROUTES

| | Subcutaneous Injection | | Intrasplenic Injection | | | | Ratio[1] |
|---|---|---|---|---|---|---|---|
| | | | Subcutaneous spleen | | Spleen, *in situ* | | |
| | Dose | Estrus | Dose | Estrus | Dose | Estrus | SC:ISSC:IS |
| | μg. | % | μg. | % | μg. | % | |
| α-Estradiol | 1.00 | 30 | 1 | 20 | 50 | 20 | |
| | 1.50 | 50 | 2 | 50 | 75 | 50 | 0.75:1:38 |
| | 2.00 | 70 | 4 | 100 | 100 | 70 | |
| α-Estradiol-3-benzoate | 0.25 | 30 | 0.50 | 40 | 15 | 30 | |
| | 0.40 | 50 | 1.00 | 50 | 25–50 | 50 | 0.4:1:40 |
| | 0.50 | 90 | 2.00 | 80 | 75 | 70 | |
| α-Estradiol-17-caprylate | 0.75 | 20 | 0.25 | 20 | 1.00 | 0 | |
| | 0.95 | 50 | 0.33 | 50 | 1.75 | 50 | 3:1:5 |
| | 1.50 | 90 | 0.50 | 80 | 2.50 | 80 | |
| α-Estradiol-3, 17-dipropionate | 0.25 | 10 | 0.30 | 10 | 5.00 | 40 | |
| | 0.40 | 50 | 0.60 | 50 | 7.50 | 50 | 0.67:1:12 |
| | 0.50 | 70 | 2.00 | 100 | 10.00 | 60 | |
| α-Estradiol-3-benzoate-17-butyrate | 2.50 | 25 | 1.00 | 30 | 2.50 | 30 | |
| | 4.00 | 50 | 2.50 | 50 | 4.00 | 50 | 1.5:1:1.5 |
| | 5.00 | 70 | 4.00 | 80 | 5.00 | 60 | |
| Estrone | 2.50 | 20 | 5.00 | 30 | 140[2] | 20 | |
| | 5.00 | 50 | 7.50 | 50 | | | 0.67:1:19 |
| | 10.00 | 90 | 10.00 | 60 | | | |
| Estrone-3-benzoate | 1.00 | 10 | 1.75 | 20 | 100 | 40 | |
| | 1.75 | 50 | 2.50 | 50 | 150 | 50 | 0.75:1:60 |
| | 2.50 | 90 | 4.00 | 80 | 200 | 90 | |
| Estriol | 12.50[2] | 0 | 12.50[2] | 0 | 12.5[2] | 0 | |
| Equilin | 5 | 10 | 15 | 40 | 100 | 30 | |
| | 10 | 50 | 20 | 50 | 125[2] | 50 | 0.5:1:6 |
| | 20 | 80 | 40 | 80 | | | |

[1] SC, subcutaneous injection; ISSC, intrasplenic injection into spleen transplanted to subcutaneous position and without portal drainage; IS, injection into spleen *in situ*.
[2] Maximum solubility.

administration for each of the estrogens employed are presented for purposes of comparison.

When free α-estradiol was assayed by this method it was necessary to use 1.5 μg. subcutaneously, 2.0 μg intrasplenically, transplanted, and 75 μg. intrasplenically *in situ* to obtain estrus in 50 per cent of the animals. The ratio for free α-estradiol therefore, is 0.75:1:38. α-

Estradiol-3-benzoate was more effective than α-estradiol by all routes of administration the ratio being 0.4:1:40. α-Estradiol dipropionate was also more effective than the free hormone by all routes of administration, but it was proportionately and absolutely more effective than the free hormone when injected into the spleen *in situ*, its ratio being 0.67:1:12. Of the various substances studied α-estradiol-17-caprylate was the most effective when injected in the spleen *in situ*, but even it was more effective by way of the subcutaneous spleen than by way of the spleen *in situ*, its ratio being 3:1:5. α-Estradiol-3-benzoate-17-butyrate was the only ester which we have studied that was less effective than α-estradiol when injected subcutaneously. Intrasplenically when the spleen was transplanted it was slightly more effective than subcutaneously and by way of the spleen *in situ* it is as effective as when given subcutaneously. The ratio for α-estradiol-3-benzoate-17-butyrate is 1.5:1:1.5.

α-Estradiol was also assayed by the same method except that the benzyl alcohol was omitted from the sesame oil. The ratio was essentially the same, namely 0.67:1:33. The absolute values for the production of estrus in 50 per cent of the animals were 1.0, 1.5, and 50 μg. by the subcutaneous, intrasplenic, transplanted, and intrasplenic, *in situ* routes, respectively.

Because of the limited solubility of estrone (140 μg. in 0.05 cc. of 5% benzyl alcohol in sesame oil) we were unable to obtain estrus in more than 20 per cent of the animals injected intrasplenically *in situ*, with a saturated solution and were unable, therefore, to arrive at a ratio of activity. Estrone-3-benzoate, like the esters of estradiol was more effective than estrone by all routes of administration. The ratio for estrone benzoate is 0.75:1:60.

Since only 12.5 μg. of estriol is soluble in 0.05 cc. of benzyl alcohol we were unable to induce estrus in any of the animals by any of the routes of administration.

Fifty per cent of the animals which received 125 μg. of equilin (maximum amount soluble in 0.05 cc. of 5% benzyl alcohol) intrasplenically, *in situ*, showed vaginal estrus. Since equilin is much less effective than estrone or estradiol by the other routes of administration it has the lowest ratio (0.5:1:6) of the free estrogens which we have studied.

In an attempt to ascertain whether prolonged estrus could be produced by the esters of estradiol administered intrasplenically 300 μg. each of α-estradiol, α-estradiol-3-benzoate and α-estradiol dipropionate were injected intrasplenically, *in situ*. Ten animals were used for each substance. The average duration of estrus was 2, 2.5 and 2 days, respectively.

## DISCUSSION

The results in animals which received subcutaneous injections of estrogens merit attention since, to our knowledge, only one other in-

vestigator, Pedersen-Bjergaard (1939) has reported that α-estradiol-3-benzoate is more effective than α-estradiol. Inasmuch as other workers used larger volumes of oil than were practicable in this investigation we have considered the possibility that the different quantities of solvent might account for the variation in results. As a test of this possibility a few experiments have been performed in which α-estradiol and α-estradiol-3-benzoate were each dissolved in relatively large volumes of oil (0.4 cc.) and injected in two portions subcutaneously. It was observed that, in general, under these conditions α-estradiol was more effective than α-estradiol-3-benzoate in our animals.

In the experiments of Emmens (1941), α-estradiol-3-benzoate proved equally effective intravaginally and less effective subcutaneously than α-estradiol. If comparison be made on the basis of the estradiol content, the ester is more effective intravaginally. Emmens states that this is probably due to an effective hydrolysis of the ester in the vagina, but it would appear equally likely that the ester acts as such on the end organ. Our observations indicate that the esters, in part at least, are absorbed without hydrolysis from the spleen. Absorption without hydrolysis of the esters is the only tenable explanation for the differences which exist in the ratio of activity of estrogens in the transplanted spleen and the spleen, *in situ*, for free α-estradiol and its esters. It is our opinion that the other possible reasons (inactivation by the spleen or variations in the rate of absorption) for the more favorable ratio shown by the esters have been eliminated by employing the subcutaneous spleen as the point of reference and the use of ratios of effectiveness rather than absolute values. Thus, when the esters are absorbed as such the hydroxyl groups are protected from the action of the liver and more effective estrogens reach the end organ. It seems reasonable to suppose that the various results which have been obtained in comparing estradiol and its esters after subcutaneous injection in oil may be explained by taking into account their different solubilities in the body fluids and the effect of differences in the volume of the vehicle on the rate of absorption. That is to say, while the less soluble α-estradiol is absorbed at a more effective rate for the induction of vaginal estrus from a large volume of oil, the more soluble esters are absorbed at a more effective rate from a small volume of oil.

The results in animals with subcutaneous spleens can be interpreted in several ways. More α-estradiol and estrone was required by this route than by subcutaneous injection. This might be due to inactivation of the hormone by the spleen, as suggested by Zondek's (1941) studies *in vitro;* or it might be the result of absorption from the highly vascular spleen at a rate so rapid as to interfere with its physiological effectiveness. The esters with the exception of α-estradiol-3-benzoate-17-butyrate and α-estradiol-17-caprylate were also less ef-

fective by this route than when given subcutaneously. Thus, it is necessary to take into account the additional possibility that the spleen is capable of hydrolyzing the esters to some extent. This possibility is also suggested by Heller's studies *in vitro*.

The results from studies with the various free estrogens are of considerable interest. The relative values for subcutaneous injection of these substances are in general agreement with other reports in which various methods of assay were used. The values for intrasplenic injections do not follow the same order of activity as those for subcutaneous injections. Equilin has the lowest ratio of activity between the transplanted spleen and the spleen *in situ* of the free estrogens studied. An explanation of this observation might be found if we accept with Doisy, *et al.*, (1942) the belief that equilin is actually a catabolic product of estrone which is destroyed in the liver to a lesser degree than estrone.

Furthermore, estrone is considerably less active than $\alpha$-estradiol by all routes, a fact which may be due to estrone's lesser peripheral activity. This interpretation of the observations on estrone would appear to oppose the concept that estrone must be converted into $\alpha$-estradiol in the liver or other tissues before it acts upon the various end organs.

The studies by Biskind (1939, 1941 a, b) in which pellets of estrogens were implanted intrasplenically show that $\alpha$-estradiol-3-benzoate is more effective than $\alpha$-estradiol.

Our own observations are in agreement with Biskind's findings with the exception that $\alpha$-estradiol-3-benzoate is also more effective than $\alpha$-estradiol both subcutaneously and in the subcutaneous spleen. The pellet studies which have been reported have failed to bring out this fact. It seems probable that this is due to the difficulty or impossibility or making pellets sufficiently small so that a maximal response is not produced in all or virtually all of the test animals when venous drainage is not into the portal system. We are now employing pellets of estrogens alone and in combination with cholesterol (as suggested by Shimkin and White (1941) in an attempt to overcome this difficulty and to study further those estrogens (estriol, estrone and equilin) whose solubility has limited their use in tests by intrasplenic injection.

In attempting to correlate our findings with those of Cantarow, *et al.*, (1942) it should be noted that their observations in no way invalidate the theory that the estrogens are inactivated in the liver. Their findings, if applicable to the situation in the rat, would only tend to indicate that more than one passage through the liver is required for the inactivation of estrogens and that this may be accomplished by an entero-hepatic circulation. There is no evidence from their work as to the effect that an interruption of this entero-hepatic circulation would have on the peripheral activity of the estrogen.

Furthermore, they used amounts of estrogen (25 mg.) which are well beyond the physiologic range. Others who have employed smaller dosages of estrogens have either failed completely to find estrogenic activity in the bile, or have succeeded in obtaining evidence of only slight activity. Heller and Heller (1943) have suggested that the biliary system may act as an overflow valve and that larger percentages of estrogen are recovered in the bile as the dosage increases.

From the results presented there is no suggestion as to how the estrogens may be inactivated. We are only able to point out that the inactivation is in some way related to the hydroxyl and ketone groups since esterification of the hydroxyl groups offers some protection against destruction. The 17-hydroxyl would appear to be much more important in this respect and the degree of protection varies with the ester used (Butenandt and Goergens, 1937; Miescher, *et al.*, 1937). This is in agreement with the findings of Miescher, *et al.* (1938 a, b, c), on the duration of estrus. They found that the 17 esters are more effective in the production of extended estrus than the corresponding 3 esters. While the inactivation may be through the medium of the production of a less active estrogen or ester it may actually be due to the disruption of the sterol nucleus. Of interest in this regard is the recent demonstration by Pearlman and Pincus (1943) that estradiol can be oxidized to estrone without protecting the OH group at the 3 position. The work of Westerfeld (1941) on the inactivation of estrone by hyrogen peroxide offers interesting speculation in this regard. He found that the fourth ring in estrone is split at the 17-ketone and a lactone is produced which has one fourteenth the activity of estrone in the spayed mouse. While it is likely that protection of the 17-group would greatly inhibit this reaction and that esterification of the 3-hydroxyl would have little effect, it should be noted that Westerfeld (1940) had reported earlier the failure of hydrogen peroxide to inactivate estrone methyl ether.

## SUMMARY

$\alpha$-Estradiol, $\alpha$-estradiol-3-benzoate, $\alpha$-estradiol dipropionate, equilin, estrone and estrone-3-benzoate are slightly less effective when injected into a transplanted spleen separated from the portal circulation than when injected subcutaneously. They are markedly less effective when injected into a spleen *in situ*. $\alpha$-Estradiol-3-benzoate-17-butyrate is equally effective when administered subcutaneously or into a spleen *in situ*. $\alpha$-Estradiol-17-caprylate and $\alpha$-estradiol-3-benzoate-17-butyrate are more effective in a transplanted spleen separated from the portal circulation than when injected subcutaneously. $\alpha$-Estradiol-17-caprylate is less effective in a spleen *in situ* than in a transplanted spleen separated from the portal circulation. The liver is a site of extensive inactivation of estrogen. The degree of inactivation is governed, in part at least, by the nature of the in-

dividual estrogen. The esters of α-estradiol are at least partially absorbed as such from the spleen. Esterification protects α-estradiol from inactivation by the liver, the 17 hydroxyl being more important than the 3 hydroxyl. The possible explanation of the results and their significance is discussed.

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# CONSTANCY OF ANDROGEN CONCENTRATION IN THE URINE

FRANKLIN HOLLANDER, BRUNO KRISS, EMANUEL KLEMPNER AND ROBERT T. FRANK

*From the Laboratories of The Mount Sinai Hospital*
NEW YORK CITY, NEW YORK

In the course of androgen bioassays performed on a consecutive series of urine specimens from each of several cases, it was noted that the daily excretion of androgens varies considerably from time to time in any one individual. On the other hand, when the androgen excretion was tabulated on a per liter basis (*i.e.*, as concentration) we observed a notable degree of constancy for any one case. This observation differs so greatly from what is generally accepted that we were led to study the relation between androgen excretion and urinary volume more closely. A review of the literature for the last five years revealed no thorough investigation of this problem. In the course of a study of the sex hormone excretion of children, Oesting and Webster (1938) reported that the quantity of androgenic substances excreted in the urine is independent of the urinary volume, a fact in keeping with the generally accepted belief. Their evidence for this was based on the absence of a graphic correlation between androgen content (in color units) of a 24-hour specimen and the urinary volume. This observation of Oesting and Webster is in harmony with the common practice of expressing urinary excretion of androgenic substances in terms of 'total amount per 24 hours' rather than in units of concentration, *i.e.*, 'amounts per liter.'

In the present investigation, we have performed a series of experiments in which the androgen excretion of 9 persons was studied over a period of about 30 days, by means of our chick-comb assay technique. The findings are contrary to those reported by Oesting and Webster, and indicate good correlation between the daily androgen output and urinary volume. The concentration of androgen, on the contrary, is relatively constant and therefore independent of the urinary volume.

## PROCEDURE

The subjects of this investigation were 2 males and 7 females, all of whom were normal from the standpoint of kidney function. Pertinent data concerning these individuals are given in the table. In 6 instances the urine was collected continuously over a period of 30 days, in ten 3-day specimens; of the other subjects, one gave eight 3-day specimens, one 9, and one 12. The urinary volume of 3 of the cases was varied during the period of observation by forcing and restricting fluid intake for several 3-day periods each.

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Each specimen of urine was extracted and assayed for androgenic activity by the chick-comb method developed in this laboratory (Frank, Klempner, Hollander and Kriss, 1942). All assays were performed within the dosage range 20–40 μg.; in this range we have found that the mean error of the method is about 13 per cent (corresponding to a standard deviation of about 16 per cent) as determined from a series of 24 determinations on

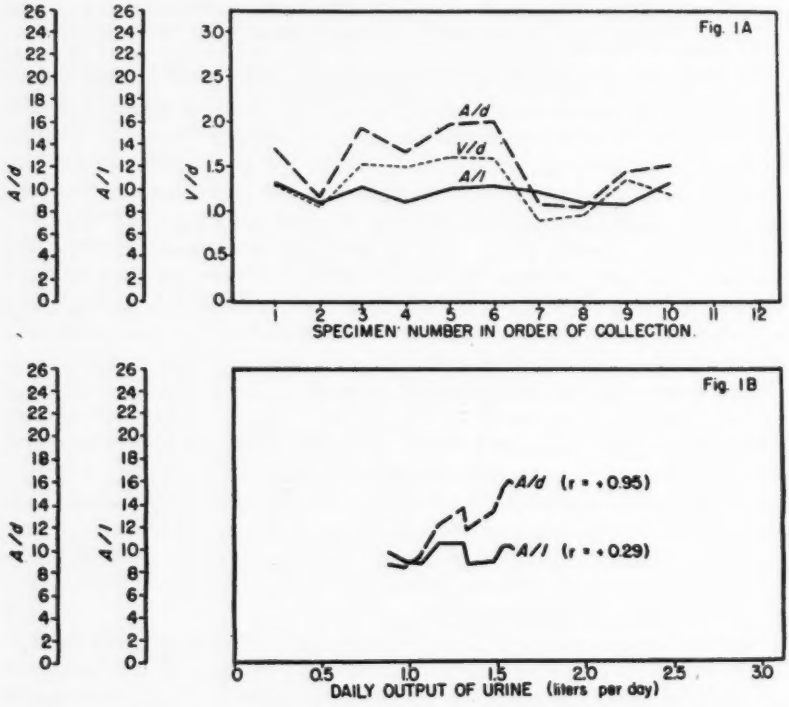


FIG. 1. Urinary androgen excretion in subject G.F.; series 41. $A/d$ = Daily androgen output, expressed as androsterone-equivalents (I.U., dmg.) per day. $A/l$ = Concentration of androgen, expressed as androsterone-equivalents (I.U., dmg.) per liter. $V/d$ = Daily urine output, expressed as liters per day. r = Coefficient of correlation.

known amounts of crystalline androsterone (Klempner, Hollander, Frank and Kriss, 1942). The results of assay were calculated in two ways, as described in our paper on technique: first, as androgen output per liter of urine (measured in decimilligrams (I.U.) of androsterone per liter), and second as androgen output per 24 hours measured in the same way.[1]

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[1] It must be kept in mind that the biological activity which we measure (as androsterone equivalents per liter, $A/l$) is actually the summated response to several different molecular types of androgen (androsterone, isoandrosterone and dehydroisoandrosterone), each of which possesses a different biological activity per unit weight of

## OBSERVATIONS

The results on one of these cases, in which fluid intake was not controlled (G.F.), is presented for illustrative purposes in figure 1. A second case, in which extreme variations in daily urinary volume were induced by careful control of fluid intake (M.S.), is presented in figure 2. A summary of all 9 cases is given in table 1.

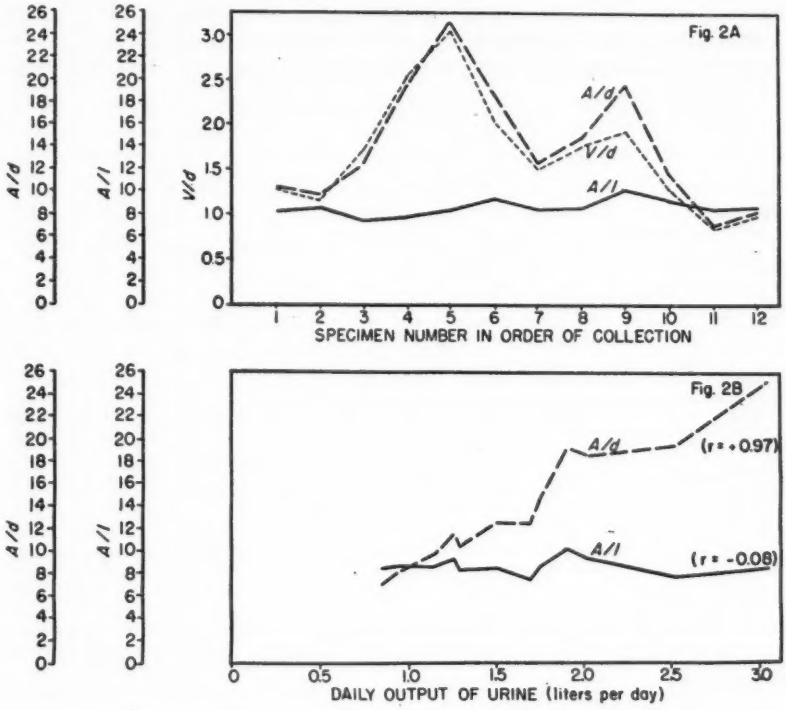


FIG. 2. Urinary androgen excretion in subject M.S., series: 52. $A/d$ = Daily androgen output, expressed as androsterone-equivalents (I.U., dmg.) per day. $A/l$ = Concentration of androgen, expressed as androsterone-equivalents (I.U., dmg.) per liter. $V/d$ = Daily urine output, expressed as liters per day. r = Coefficient of correlation.

Figure 1A shows the graphs for androgen excretion in terms of concentration of androgen per liter ($A/l$), and in terms of daily output of androgens ($A/d$), as well as the urinary volume in liters per day ($V/d$), all three of these being plotted against the number of the

substance. Hence, the unit 'androsterone equivalent per liter' is actually an average measure of activity-concentration, rather than of concentration of chemical substance. This unit has been defined by us as the number of decimilligrams (dmg.) of crystalline androsterone which yield the same adjusted comb growth response as the quantity of urine or other extract employed in the assay. The decimilligram was used as a measure of weight because it corresponds to the international unit of androgenic activity.

specimen in the order of collection. The following facts are indicated: *a*) Since the curve for androgens per liter is practically a straight horizontal line, it may be inferred that the concentration of biologically active androgens in the urine is relatively constant throughout the period of observation. The range of variation of these 9 concentration values is 8.6 to 10.4 I.U. (dmg.) per liter, with a standard deviation (S.D.) of $\pm 0.70$ I.U. (dmg.) per liter, equivalent to 7.3 per cent of the mean value. This percentage variability is less than the percentage variability (*i.e.*, the reliability) of the assay method itself.[2] *b*) The curve for androgens per day, on the contrary, shows marked and irreg-

TABLE 1. SUMMARY OF THE DATA ON ANDROGEN EXCRETION IN NINE SUBJECTS

| Subject | F.H. | E.K. | R.B. | G.F. | M.S.[1] | P.S. | A.B. | E.C. | Y.M. |
|---|---|---|---|---|---|---|---|---|---|
| Sex | M | M | F | F | F | F | F | F | F |
| Age (yr.) | 41 | 33 | 29 | 36 | 24 | 30 | 30 | 20 | 37 |
| Diagnosis[2] | N | N | N | N | O | 2°A | 1°A | H | TAO |
| Control of fluid intake | Yes | Yes | No | No | Yes | No | No | No | No |
| No. of 3-day specimens | 10 | 10 | 10 | 10 | 12 | 10 | 10 | 9 | 8 |
| $V/d$[3] (mean) | 1.42 | 1.11 | 0.90 | 1.29 | 1.66 | 1.23 | 1.50 | 0.69 | 1.20 |
| S.D.[4] | 0.69 | 0.35 | 0.20 | 0.25 | 0.62 | 0.21 | 0.30 | 0.04 | 0.18 |
| S.D. (%) | 48.6 | 31.5 | 22.2 | 19.4 | 37.3 | 17.1 | 20.0 | 5.8 | 15.0 |
| $A/l$[5] (mean) | 18.0 | 32.7 | 10.1 | 9.6 | 8.5 | 8.7 | 8.3 | 10.1 | 5.4 |
| S.D. | 2.97 | 4.91 | 1.31 | 0.70 | 0.67 | 0.82 | 1.06 | 1.18 | 0.44 |
| S.D. (%) | 16.5 | 15.0 | 12.9 | 7.3 | 7.9 | 9.5 | 12.8 | 11.7 | 8.1 |
| $A/d$[6] (mean) | 24.6 | 35.2 | 9.0 | 12.4 | 14.0 | 10.7 | 12.3 | 6.9 | 6.6 |
| S.D. | 11.24 | 7.31 | 2.16 | 2.79 | 5.23 | 2.45 | 2.54 | 1.01 | 1.47 |
| S.D. (%) | 45.6 | 20.8 | 24.0 | 22.5 | 37.3 | 22.8 | 20.6 | 14.6 | 22.3 |
| r for $A/l$ and $V/d$ | −0.31 | −0.55 | −0.22 | +0.29 | −0.08 | +0.48 | −0.20 | −0.52 | +0.92 |
| r for $A/d$ and $V/d$ | +0.95 | +0.78 | +0.88 | +0.95 | +0.97 | +0.95 | +0.85 | +0.72 | +0.99 |

[1] Confined to bed in the hospital.
[2] Diagnoses: N = Normal; O = Osteochondritis of the spine; 2°A = Secondary amenorrhea; 1°A = Primary amenorrhea; H = Hirsutism; TAO = Thrombo-angeitis obliterans.
[3] Urine volume per day.
[4] S.D. = Standard deviation.
[5] Androgen excretion per liter (I.U.).
[6] Androgen excretion per day (I.U.).

ular variations around the horizontal; the range is 8.4 to 16.0 I.U. (dmg.) per day, with a standard deviation of $\pm 2.79$, equivalent to 22.5 per cent of the mean. This measure of variability is about 3 times as great as that for the concentration data, which indicates a consider-

[2] The reliability of the assay method as established on a series of 39 knowns in the androsterone dosage range of 10–50 micrograms corresponded to a mean error of 25 per cent. On the basis of 24 of these assays, in the dosage range 20–40 micrograms, the mean error was 13 per cent. For assays performed in an even narrower dosage range (*e.g.*, 25–35) the error would be correspondingly less, and this was actually the case in the urine assays here reported. Furthermore, in a series of 9 assays the frequency is so low that the S.D. of such a distribution might show a considerable divergence from the S.D. of the parent population (or even from that of another sample population) without the difference in the values for S.D. being statistically significant.

able degree of variability in the same urine specimen when studied from the point of view of amount excreted per day instead of amount per liter. In order to determine whether the extensive variations in the data on the daily output of androgens are correlated with the daily output of urine ($V/d$), we plotted these variables against each other (fig. 1B). The graph gives fairly good evidence that increasing values of androgens per day tend to be associated with increasing values for the volume of urine per day. From the analogous curve for the androgen content per liter, however, it is evident that such a correlation between this variable and the output of urine does not exist; in fact, the horizontal nature of the graph for androgens per liter indicates that the androgen output in terms of concentration is constant irrespective of the urinary output. The correlation coefficients (r) for these 2 sets of data are +0.95 for androgens per day against the urine volume per day, and +0.29 for androgens per liter against urine volume per day, which clearly confirms the graphic evidence.

An even more striking picture is afforded by case M.S., in which extreme variations in urinary volume were experimentally produced. In this subject, the output varied between 2.53 and 9.16 liters per 3-day period, in contrast with a range of 2.65 to 4.77 for the previous case (G.F.) whose fluid intake was uncontrolled. Examination of figure 2A reveals a similar horizontal line for androgens per liter ($A/l$) as in case G.F., and a graph for androgens per day with even more marked variations than in the previous case. The range of values for androgens per liter is 7.32 to 10.07 I.U. (dmg.) per liter (S.D. = $\pm$0.67 or 7.9%), whereas for androgens per day the range is 6.98 to 25.05 I.U. (dmg.) per day (S.D. = $\pm$5.23 or 37.3 per cent). In terms of the percentage values, the data for androgens per day are almost 5 times as variable as the values for androgens per liter. By plotting each of these variables against the daily output of urine (fig. 2B) we see that the evidence for correlation of androgens per day ($A/d$) and urine volume per day ($V/d$) is even more striking than in figure 1B, because of the greater range of urinary output. The data for androgens per liter, on the other hand, manifest the same degree of constancy as in the first case. The correlation coefficients for these 2 curves are +0.97 for androgens per day against urine volume per day, and −0.08 for androgens per liter against urine volume per day.

A summary of all 9 cases as presented in table 1 shows that the data for androgens per day are uniformly more variable than those for the androgens per liter. The standard deviation for the former is never less than 14.6 per cent and in one case it reaches a high of 45.6 per cent, whereas for the latter it ranges between 7.3 and 16.5 per cent. Correspondingly, r-values for androgens per liter against urine volume per day are less than 0.6 (in absolute value) in all cases except Y.M., for which r = 0.92. For androgens per day against urine volume per day, r is never less than 0.7 and is 0.95 or higher in 5 of the 9 cases.

## DISCUSSION

Our data give satisfactory evidence that there exists a high correlation between the daily output of biologically active androgenic substances and the daily output of urine, for each of the 9 individuals studied. On the other hand, no such correlation exists between the concentration of androgens and the urinary output. In fact, the data expressed in concentration units (I.U. per liter) are relatively constant throughout the entire period of observation which was sometimes as long as 36 days, whereas the data expressed in units of quantity (I.U. per day) show variations which, in some instances, are 4 to 5 times as great as the concentration data. Even wide variations in urinary volume, produced by the experimental restriction and increase of fluid intake, resulted in correspondingly augmented changes in the daily excretion of androgens but produced no such variations in their concentration.

In addition to the disagreement with the report of Oesting and Webster, our findings differ from what might have been expected. In general, the concentration of urinary constituents is decreased as the volume of urine increases (*i.e.*, the correlation is negative), whereas in 6 of our 9 cases, the reverse actually occurred as shown by positive correlation coefficients.[3] The variations in concentration values of any one case which do exist are never greater than might be expected from the reliability of the bioassay method itself. It is possible that a more precise method of determination of androgens would reveal an even greater degree of constancy of the concentration data, but this remains to be demonstrated. It must be remembered, also, that all of the foregoing observations are concerned only with biologically active androgenic material. Whether similar generalizations obtain for chemically related but biologically inactive ketosteroids, as determined colorimetrically, is a matter for further investigation.

So far as we know, no urinary constituent is present in a constant concentration, independent of the volume of fluid excreted. Hence, the foregoing results present a unique situation in kidney physiology. We are aware of the singularity of these results, but we cannot overlook the fact that all 9 cases studied, and especially the 3 with experimentally varied fluid output, are consistent in this respect. We are unable to offer any explanation for this observation, although some explanation in harmony with general renal physiology may become apparent when a method for determining the androgen level in blood becomes available. The immediate implication of such constant concentration of androgen content in the bladder urine is that the concentration of androgen activity is the same in the glomerular filtrate and in the fluid reabsorbed by the tubules, thus leaving the concen-

[3] Some of these positive coefficients are of questionable statistical significance because of the small number of specimens in any one case, but this means only that they do not differ significantly from zero.

tration in the tubular urine unchanged. Any further interpretation of the kidney mechanism which might cause such a constancy of concentration is premature.

Since the total daily output of androgen increases with the daily output of urine, any considerable increase in fluid output may exert a depleting effect on the androgen content of the body. Whether this is so, however, will depend on the total content and rate of formation of androgen in the body, relative to the quantity being excreted.

## SUMMARY

Each of 9 human subjects, normal from the standpoint of kidney function, was studied by determining the biologically active androgen content and urinary volume of a series of consecutive 3-day specimens of urine, collected for a period of approximately one month. In 3 of these cases, the urinary output was varied throughout the period of study by forced and restricted fluid intake. Androgen content was determined by the chick-comb method previously described by us, and the results were expressed in terms of *a*) output per liter of urine (concentration) and *b*) output per 24 hours.

The results show that the concentration of biologically active androgens in the urine is constant within the limits of reliability of the assay method and independent of the daily urinary output, even under forced and restricted fluid intake. The standard deviation for any one set of values never exceeded 16.5 per cent of the mean. Contrary to the foregoing, the androgen output per 24 hours shows considerable variation; the standard deviation for any one set of these values was invariably greater than that for the concentration data, the difference being as much as 4.5-fold in an extreme case. However, these variations in daily output of androgens were not irregular but paralleled the daily output of urine. The correlation coefficients were 0.95 or greater in 5 of the 9 cases, and never fell below 0.72. This finding is contrary to the report of Oesting and Webster. Such constancy of concentration is not in accord with the behavior of any other urinary constituent. Further study of this problem is necessary before any definite conclusion can be drawn concerning kidney function in relation to androgen excretion.

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# STUDY OF INTERRELATIONSHIP OF PANCREATIC DIABETES WITH ENDOCRINE GLANDS IN THE TOAD

CHRISTIANE DOSNE[1]

*From the Institute of Physiology, Faculty of Medical Sciences*
BUENOS AIRES, ARGENTINA

THE production of diabetes in the toad, *Bufo arenarum Hensel*, by extirpation of the pancreas, as well as the modifications brought about by simultaneous extirpation of other glands have been extensively studied by Houssay and his collaborators (1930a, b). Pancreatectomy in all cases was performed by ligating and cutting the blood vessels from the pancreas to the stomach; the portion of the pancreas next to the liver was ligated and cut close to the latter; tissue adhering to the duodenum was separated with scissors, and that adhering to the portal vein and its branches was ligated and cut. This rather drastic procedure assured the complete removal of the pancreas, but the circulation to the liver and stomach was so impaired that 90 per cent of the animals died within 4 to 8 days after the operation (Foglia, 1942).

A new operative procedure has been developed recently in which there was no damage to the circulation and almost no mortality while a prolonged diabetic state was obtained. Under light ether anesthesia, the pancreas was brought out through a left ventral incision. The little finger was slipped under the hepatic vessels, and the latter were rubbed free of pancreatic tissue, using dry cotton pellets held in forceps; care was taken not to damage the vessels. In a similar manner, the pancreas was removed from the vessels to the stomach and duodenum and from the portal vessels with very little hemorrhage. By this method, a small amount of pancreatic tissue adheres to the hepatic vessels and to the duodenum, but it does not prevent the early appearance of hyperglycemia while the intact circulation to the liver and stomach assures an almost 100 per cent survival of the animals.

In table 1 a comparison is made of the time of onset and the degree of intensity of hyperglycemia following complete and subtotal pancreatectomy. Each group contained a large number of animals in order to compensate for the wide individual variations characteristic of toads.

Twenty-four hours after the operation there was definite evidence of hyperglycemia in animals in both groups although the blood-sugar

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values were higher in the group undergoing total pancreatectomy. Forty-eight hours after the operation the blood-sugar level was the same in both cases. The totally pancreatectomized toads did not survive more than 5 days. The partially pancreatectomized animals were still diabetic and in good shape at the end of 40 days.

This and all of the experiments reported herein were made during the winter months. It was found that during the summer, subtotally pancreatectomized toads would either die in the diabetic state within one month or show a disappearance of the diabetes. This latter could be accounted for histologically by the hyperplasia of the islets of Langerhans. Regeneration of the remnants of pancreas was never encountered during the winter season. Such seasonal variations

TABLE 1. EFFECT OF TOTAL AND OF SUBTOTAL PANCREATECTOMY IN THE TOAD ON THE BLOOD-SUGAR LEVEL

| Days after Operation | Unoperated Controls | | Subtotal Pancreatectomy | | Total Pancreatectomy | |
|---|---|---|---|---|---|---|
| | Blood sugar | No. of toads | Blood sugar | No. of toads | Blood sugar | No. of toads |
| | *mg. %* | | *mg. %* | | *mg. %* | |
| 1 | 30 (23–41) | 6 | 52 (39–73) | 10 | 78 (61–95) | 10 |
| 2 | 32 (24–38) | 8 | 88 (50–139) | 8 | 82 (43–112) | 9 |
| 5 | 30 (23–41) | 6 | 108 (35–213) | 9 | 83 (19–155) | 9 |
| 15 | 20 (14–29) | 6 | 78 (15–167) | 6 | | |
| 25 | 27 (20–36) | 6 | 83 (39–150) | 8 | | |
| 30 | 29 (24–35) | 7 | 50 (43–60) | 7 | | |
| 40 | 33 (27–36) | 7 | 68 (50–104) | 7 | | |

are characteristically observed in the toad, as was shown by Mazzocco (1938a, b).

The diabetes which follows subtotal pancreatectomy, just as that following total pancreatectomy, can be intensified by the injection of anterior pituitary lobes (Houssay and Biasotti, 1930a, b). Forty-eight hours after partial pancreatectomy, three groups of toads were subcutaneously injected with 1, 2 and 5 anterior pituitary lobes, respectively; the tissue had been triturated in physiological saline so that each animal received 1 cc. of a suspension. Twenty-four hours later all animals were killed by destruction of the medulla and blood was drawn from the heart for blood-sugar determinations. These results are summarized in table 2. Anterior pituitary tissue has no effect on the blood sugar of unoperated toads while it causes a progressive increase in the already diabetic animals. Two intact toads which received 54 lobes each in 2 days, 24 hours later had an average blood sugar of 67 mg. per cent as compared with 63 mg. per cent in the non-treated controls, the normal blood sugar level being higher due to the summer season.

TABLE 2. EFFECT OF INJECTION OF ANTERIOR-PITUITARY LOBES ON BLOOD SUGAR IN NORMAL AND OPERATED TOADS

| No. of Anterior Lobes Injected | Unoperated Controls | | Subtotal Pancreatectomy | |
|---|---|---|---|---|
| | Blood sugar | No. of toads | Blood sugar | No. of toads |
| | *mg. %* | | *mg. %* | |
| 0 | 34 (28–46) | 6 | 62 (40–95) | 6 |
| 1 | 37 (26–41) | 6 | 99 (71–126) | 6 |
| 2 | 32 (24–38) | 8 | 108 (57–171) | 9 |
| 5 | 35 (26–41) | 6 | 159 (64–226) | 6 |

There is a remarkable decrease in the severity of the symptoms of pancreatic diabetes following extirpation of the anterior lobe of the hypophysis with re-establishment of the diabetes upon implantation of anterior lobes (Houssay and Biasotti, 1930a, b). The data in table 3 show that 5 and 15 days after subtotal pancreatectomy removal of the anterior lobe (pars distalis) caused a disappearance of the diabetes and that upon the subcutaneous injection of 2 anterior lobes per animal, 24 hours before killing them, there was a return of the hyperglycemia.

TABLE 3. BLOOD SUGAR LEVELS IN TOADS FOLLOWING SUBTOTAL PANCREATECTOMY AND HYPOPHYSECTOMY

| Time after Operation | 5 Days | | 15 Days | |
|---|---|---|---|---|
| | Blood sugar | No. of toads | Blood sugar | No. of toads |
| | *mg. %* | | *mg. %* | |
| Unoperated controls | 23 (21–26) | 6 | 23 (14–39) | 6 |
| Hypophysectomy | 19 (9–32) | 10 | 15 (12–21) | 6 |
| Pancreatectomy | 104 (54–162) | 10 | 65 (51–71) | 6 |
| Hypophysectomy & Pancreatectomy | 23 (14–41) | 10 | 25 (22–31) | 6 |
| Hypophysectomy & Pancreatectomy +2 Anterior Lobes | 82 (23–139) | 10 | 58 (47–74) | 6 |

It has been demonstrated by Long and Lukens (1934, 1936) in the cat and dog, and by Houssay and Biasotti (1936a, b) that the adrenal cortex may have an effect similar to that of the anterior pituitary upon the diabetes; that is, upon suppression of adrenal function there is an attenuation of the diabetes. Houssay and Biasotti further observed that the diabetes re-appeared upon injection of anterior pituitary lobes, even in animals deprived of adrenals, anterior pituitary and pancreas. Following subtotal pancreatectomy and adrenalectomy (performed by diathermo-coagulation) there was attenuation of the diabetes as shown by the results obtained on blood samples 36 and 48 hours after the double operation (table 4). The injection of anterior lobes restored the diabetic state in spite of the absence of the adrenals.

The effect of the thyroid gland on pancreatic diabetes is still the subject of debate. In toads, thyroidectomy was performed by cutting

away the hyoid bone to which the thyroid gland adheres. When this operation was performed simultaneously with a subtotal pancreatectomy and the animals were killed at the end of 5 days, the blood-sugar values were most irregular. In some cases there was marked hyperglycemia while in others there was evident hypoglycemia. These irregularities in blood-sugar levels, curiously enough, were also observed in animals in which a sham thyroidectomy had been performed. In order to prevent the effects produced by surgical interference in the

TABLE 4. BLOOD SUGAR LEVELS IN TOADS FOLLOWING SUBTOTAL PANCREATECTOMY AND ADRENALECTOMY

| Time after Operation | 36 Hours | | 48 Hours | |
|---|---|---|---|---|
| | Blood sugar | No. of toads | Blood sugar | No. of toads |
| | *mg. %* | | *mg. %* | |
| Unoperated controls | 32 (24–38) | 8 | 34 (30–41) | 6 |
| Adrenalectomy | 24 (15–31) | 8 | 24 (17–31) | 6 |
| Pancreatectomy | 55 (28–117) | 8 | 83 (60–121) | 6 |
| Adrenalectomy & Pancreatectomy | 43 (33–51) | 8 | 33 (19–42) | 8 |
| Adrenalectomy & Pancreatectomy & 2 Anterior Lobes | 67 (42–149) | 8 | 108 (49–171) | 9 |

region of the thyroid, effects which remain unexplained, thyroidectomy was performed a month or more before the pancreatectomy. In this way it was possible to show that extirpation of the thyroid in itself in no way modifies either the normal blood sugar or the production of pancreatic diabetes (table 5). In a similar way it was found

TABLE 5. BLOOD-SUGAR LEVELS IN TOADS FOLLOWING SUBTOTAL PANCREATECTOMY AND THYROIDECTOMY

| Time after Thyroidectomy, Pancreatectomy | 30 Days 5 Days | | 45 Days 5 Days | |
|---|---|---|---|---|
| | Blood sugar | No. of toads | Blood sugar | No. of toads |
| | *mg. %* | | *mg. %* | |
| Unoperated controls | 31 (20–45) | 10 | 42 (37–53) | 6 |
| Thyroidectomy | 41 (20–70) | 10 | 42 (28–61) | 10 |
| Pancreatectomy | 63 (45–127) | 10 | 63 (51–76) | 6 |
| Thyroidectomy & Pancreatectomy | 51 (30–122) | 10 | 66 (44–101) | 9 |

that the simultaneous injection of 1 mg. of thyroxine with 3 toads' anterior lobes into pancreatectomized animals produced a hyperglycemia which was not significantly different from that obtained with anterior lobes alone. Twenty pancreatectomized toads receiving 3 anterior pituitary lobes each, had blood-sugar values of 191 (123–282) mg. per cent while 20 pancreatectomized animals receiving 1 mg. of thyroxine in addition to the 3 anterior pituitary lobes had blood-sugar values of 163 (98–243) mg. per cent; 20 pancreatectomized controls had values of 99 (46–185) mg. per cent.

SUMMARY

A technique for subtotal pancreatectomy in toads has been devised following which there developed a prolonged state of diabetes, when the operation was performed during the winter season; during the summer months the blood sugar returned to its normal level due to the regeneration of the remaining pancreatic tissue. The advantage of this method of subtotal over total pancreatectomy is evident since there is no intereference with the systemic circulation and the animals may survive for as long as 40 days. Simultaneous removal of the anterior lobe of the hypophysis prevented the appearance of pancreatic diabetes, while subsequent injection of anterior pituitary tissue provoked hyperglycemia. Anterior pituitary tissue does not produce hyperglycemia in intact toads showing that in this species, unlike the dog, the pancreas is strongly antidiabetogenic. Adrenalectomy also attenuates the state of pancreatic diabetes, and the latter can be made to reappear upon the injection of anterior pituitary lobes. Extirpation of the thyroid on the other hand, in no way modifies the production of pancreatic diabetes.

I am greatly indebted to Prof. B. A. Houssay, under whose supervision this work was done.

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# A STUDY OF THE COLORIMETRIC ASSAY OF URINARY 17-KETOSTEROIDS[1]

WILLIAM W. ENGSTROM AND HAROLD L. MASON

*From the Mayo Foundation*

ROCHESTER, MINNESOTA

AT present two technics based on the Zimmermann (1935, 1936) color reaction are widely used for the determination of urinary 17-ketosteroids. In the procedure of Callow (1938) absolute alcohol is used as the solvent for the steroid solution, for the 2 per cent *m*-dinitrobenzene and for the 2.5 N potassium hydroxide. Holtorff and Koch (1940) used 95 per cent alcohol for the solutions of steroid and *m*-dinitrobenzene but an aqueous solution of 5 N potassium hydroxide. The latter workers considered that a 5 N solution of alkali gave optimal results. Since no direct comparisons of the results obtained by these two methods have been made, it was thought advisable to investigate the accuracy of each method and to determine, as far as possible, the reasons for any discrepancies noted.

Application of the colorimetric assay to crude extracts of urine meets with two difficulties, *a*) the presence of colored substances which absorb in the green region and *b*) the presence of chromogens other than 17-ketosteroids. In the method of Holtorff and Koch (1940) and in other methods in which aqueous solutions of alkali are employed, a simple blank consisting of alkali and the extract has been used to compensate for the colored substances present. However, if chromogens other than 17-ketosteroids are present such a correction would not be adequate. Evidence will be presented to substantiate this conclusion.

Fraser and coworkers (1941) using Callow's method of assay, suggested the use of a correction equation to compensate for the overestimation that results from the presence of unknown chromogens when the intensity of the red color is measured with the green filter. Callow (1938) and Talbot (1940a) showed that the colored substances and chromogenic material other than 17-ketosteroids present in crude extracts absorb maximally at 4100 Å (violet). The color produced by solutions of crystalline 17-ketosteroids absorbs maximally at 5200 Å (green). To apply a correction equation two fundamental requirements must be fulfilled: the points of maximal absorption of the pigments must be known and the colored products must follow Beer's law. The theoretical considerations, on which the formula is based, are given in Gibson's and Evans' article (1937) in which a similar problem was involved. By making readings with green and violet filters and em-

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ploying the equation a corrected green reading can be obtained. The equation is as follows: Corrected green reading $= K_i E_G - E_V / K_i - K_s$.

$E_G$ and $E_V$ are the extinctions obtained with the use of the green or violet filters respectively. $K_i = E_V / E_G$ for the unknown chromogens $K_s = E_V / E_G$ for pure 17-ketosteroid.

When a Klett-Summerson (Summerson, 1939) colorimeter is used, the readings $V$ and $G$ can be substituted for $E_V$ and $E_G$ since the scale is plotted logarithmically and the extinction, $E$, is proportional to the reading. At this point certain ambiguities in the study by Talbot, Berman and MacLachlan (1942a) should be clarified. Although they used the same form of the equation as Fraser and associates (1941), the meanings of the $K_i$ and $K_s$ constants were the reciprocals of the ones that should have been applied as Talbot acknowledged in a footnote to a subsequent article (1942b). However, further clarification is necessary since the values given for $K_i$ and $K_s$ only can be applied to another form of this equation: Corrected green reading $= K_s(E_G - K_i E_V) / K_s - K_i$, where $K$ is $E_G / E_V$.

Talbot and his coworkers (1942a), applying Callow's method to the crude extract and apparently using the proper equation, concluded, however, that the use of this equation was generally justifiable in that the corrected results thus obtained agreed with those obtained directly with the separated ketonic fractions. Our results agree with such a conclusion and also suggest that the chromogens, not 17-ketosteroid, are largely nonketonic in nature and can be eliminated by isolation of the ketonic fraction with Girard's reagent. Although our results, as well as those of Talbot and associates (1942a) indicate that this suggestion is only approximately correct, the ketonic fraction is as close as can be approached at present to the pure 17-ketosteroids. This is shown by the fact that the $V/G$ ratio obtained for the ketonic fraction approximates, but is not identical with, that of the crystalline sterone (the $K_s$ value). Consequently, it was thought that recovery experiments, made by adding solutions of crystalline steroid to crude extracts and nonketonic fractions, would be a better means of ascertaining the accuracy of the correction equation. The results so obtained would indicate that the use of the equation is justifiable.

The investigations of Friedgood (1941) and of Strickler and coworkers (1941) showed that the use of aqueous solutions of alkali led to a lack of proportionality between the intensity of the red color developed and the amount of crude extract used. This conclusion is also supported by our results with the Holtorff-Koch method. The existence of such a lack of proportionality would be a definite detriment to any colorimetric determination and it would be anticipated that, if the deviation from linearity were of sufficient magnitude, the use of a correction equation would not be valid. Data will be presented to substantiate this conclusion.

In an earlier communication Talbot and coworkers (1940b), using

Callow's method of assay, suggested that more accurate results can be obtained by assaying the ketonic fraction separated from the crude extract. Since it might be anticipated that the Holtorff-Koch technic would not give accurate results with the crude extract, it was thought advisable to determine whether, with the removal of the majority of the chromogens in the nonketonic fraction, the assay of the ketonic fraction would give the same results as with the Callow technic. It was found that the ketonic fraction yielded practically identical results by both methods while the crude extracts did not. The apparent reason for this discrepancy will be presented.

## PROCEDURES

Crude extracts of urine were prepared by the method described by Talbot and coworkers (1940a) using independent hydrolysis and extraction. The extracts were taken up in appropriate amounts of absolute alcohol. For the assay, the absolute alcohol technic of Callow and coworkers (1938, 1939) was followed, using green (maximal transmission at 5200 Å) and violet (maximal transmission at 4200 Å) filters. A standard solution of dehydro-iso-androsterone was run simultaneously in order to obviate any error which might occur with the use of a calibration curve and in order to give accurate information as to the ratio of the violet to green readings for the crystalline steroid ($K_s$).

Also, the preparation of reagents and procedure for assay were carried out as directed by Holtorff and Koch (1940), using an extract-potassium hydroxide blank in an effort to compensate for interfering pigments remaining in the extract.

A reaction time of 90 minutes was used with the aqueous alkali method since maximal color development is attained at about 75 minutes and is maintained at a plateau for at least 105 minutes. With the Callow method, maximal color is developed within 45 minutes. Our observations indicate that under these circumstances androsterone and dehydro-iso-androsterone give equal amounts of color within the limits of experimental error. Pincus and Pearlman (1941), using the Holtorff-Koch technic and allowing 45 minutes for the reaction to occur, stated that androsterone and dehydro-iso-androsterone give "almost the same" color titer.

Finally, aliquots of the alcoholic crude extracts were separated into ketonic and nonketonic fractions with Girard's reagent-T, according to the method described by Talbot and coworkers (1940b). The separated fractions were taken up in absolute alcohol.

## RESULTS AND DISCUSSION

The value of $K_i$ was determined with the Callow technic for the nonketonic fractions separated from nineteen different crude extracts. $K_i$ varied from 1.8 to 1.3, with an average of 1.5. Since 12 of the 19 values for $K_i$ deviated from the average value by not more than 0.1, the possible error which may result from use of the average value for any particular crude extract will usually be no larger than other errors involved in the actual determination. This conclusion is supported by data to be presented which demonstrate agreement between

the corrected values for the crude extract and the values obtained for the corresponding ketonic fractions. When the Holtorff-Koch method was applied to 8 nonketonic fractions, the same range of values and average for $K_1$ were obtained as with the Callow method. $K_2$ was determined each time the assay was performed; the value by the Callow method was about 0.4 and with the Holtorff-Koch method about 0.6.

In order to convince ourselves of the validity of the correction equation in our hands, studies were made similar to those of Talbot and his associates. For these we used 26 different crude extracts and

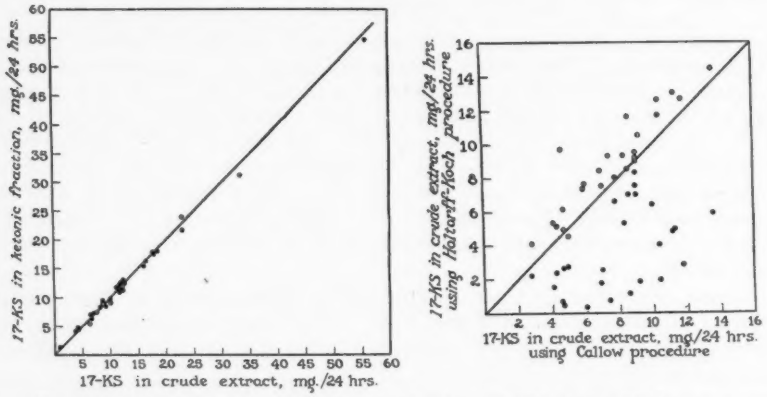

17-KS in ketonic fraction, mg./24 hrs.
17-KS in crude extract, mg./24 hrs.
17-KS in crude extract, mg./24 hrs. using Holtorff-Koch procedure
17-KS in crude extract, mg./24 hrs. using Callow procedure

FIG. 1 (*left*). Comparison of the corrected values of 17-ketosteroids for crude extracts with the values for the corresponding ketonic fractions. The straight line represents the milligrams of 17-ketosteroid determined in the crude extract with Callow's method and corrected by means of the equation. The dots locate the values found for the corresponding ketonic fractions with Callow's method and the circles those determined by the Holtorff-Koch method.

FIG. 2 (*right*). Comparison of the Callow and Holtorff-Koch procedures for the determination of the 17-ketosteroid content of crude extracts. The straight line represents the milligrams of 17-ketosteroid determined in the crude extract with Callow's method and corrected by means of the equation. The circles locate the values found for the same crude extracts with the Holtorff-Koch method and corrected by means of an extract-KOH blank. The dots locate those obtained with the Holtorff-Koch method and corrected with the equation.

the ketonic fractions separated from them. When Callow's method was used, correction of the readings by means of the equation gave values for the crude extracts, which agreed closely with the results obtained with the ketonic fractions. The difference between the two values did not exceed 10 per cent and the average difference was only 4 per cent. The results are summarized in figure 1.

As mentioned previously, the values obtained with the ketonic fraction were assumed to be correct and other values were compared with these as the standard. Certainly this fraction should contain only the ketones present in the crude extract but the validity of the assumption may be questioned when the possible presence of carbonyl

compounds which are not 17-ketosteroids is considered. The Zimmerman reaction is not specific for 17-ketosteroids although, as Callow has shown, many ketones give a faint color or the color fades rapidly. The presence of small amounts of carbonyl derivatives other than 7-ketoseroids is indicated by the $V/G$ ratios (0.40–0.58) of the ketonic fractions which approached, but were not identical with, the $V/G$ ratio ($K_s$) of dehydro-iso-androsterone. In any event the ketonic fraction is the only standard for comparison available and since its $V/G$ ratio approaches that of dehydro-iso-androsterone, it is a fairly safe assumption that it consists mainly of 17-ketosteroids.

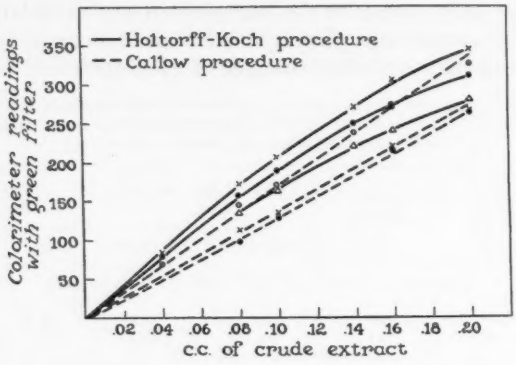
TABLE 1. RECOVERY OF CRYSTALLINE DEHYDRO-ISO-ANDROSTERONE ADDED TO NONKETONIC AND CRUDE EXTRACTS. THE CORRECTION EQUATION AND THE CALLOW PROCEDURE WERE EMPLOYED

| Sample | 17-Ketosteroid in Crude Extract, μg. | Pure Steroid Added, μg. | Total 17-Ketosteroid, μg. | | Pure Steroid Recovered | |
|---|---|---|---|---|---|---|
| | | | Calculated | Determined | μg. | Per cent |
| *Crystalline steroid plus different crude extracts* | | | | | | |
| 1 | 19.3 | 40 | 59.3 | 57.9 | 38.6 | 96 |
| | 16.9 | 60 | 76.9 | 74.3 | 57.4 | 96 |
| 2 | 17.3 | 40 | 57.3 | 55.1 | 37.8 | 95 |
| | 15.1 | 60 | 75.1 | 71.5 | 56.4 | 94 |
| 3 | 23.1 | 40 | 63.1 | 61.6 | 38.5 | 96 |
| | 20.2 | 60 | 80.2 | 78.8 | 58.6 | 98 |
| 4 | 24.8 | 40 | 64.8 | 63.0 | 38.2 | 96 |
| | 21.6 | 60 | 81.6 | 81.0 | 59.4 | 99 |
| *Crystalline steroid plus different nonketonic fractions* | | | | | | |
| 1 | | 60 | | | 57.3 | 96 |
| 2 | | 60 | | | 59.9 | 100 |
| 3 | | 60 | | | 59.9 | 100 |
| 4 | | 60 | | | 60.1 | 100 |
| 5 | | 60 | | | 58.4 | 97 |
| 6 | | 60 | | | 63.2 | 105 |
| 7 | | 60 | | | 61.1 | 102 |

It appeared to us that the validity of the correction equation could be more firmly established by a study of its use in recovery experiments. Consequently known amounts of crystalline dehydro-iso-androsterone were added to various crude extracts and to nonketonic fractions; the determinations were made with Callow's method and the correction applied. The data so obtained are shown in table 1. The maximal error in recovery for any sample was 6 per cent. We have thus confirmed results of Talbot and his coworkers on the use of the equation and have further extended them by showing that the use of the equation permits satisfactory recovery of 17-ketosteroid added to crude extracts and to nonketonic fractions. Although it is possible that an occasional specimen of urine would contain some substance which would interfere more than in any of the specimens

which we have encountered, we conclude that the correction equation is generally applicable when the Callow method is employed.

Since the Callow method gave practically identical results for the ketonic fractions and crude extracts when the correction equation was used, these results were compared with those obtained by the use of the Holtorff-Koch procedure. As stated previously, it should not be



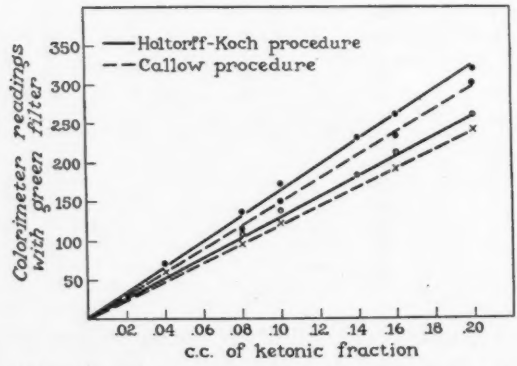




FIG. 3 (*above*). Relation between amount of crude extract and colorimeter reading with the green filter.

FIG. 4 (*below*). Relation between amount of ketonic fraction and colorimeter reading with the green filter.

possible to compensate adequately for the chromogens which are not 17-ketosteroids by using the simple blank consisting of crude extract and alkali since the dinitrobenzene is absent from the reaction mixture. The data given in figure 2 substantiate this conclusion. When such a blank was utilized, a few of the results obtained agreed with the corrected results obtained with the Callow method. Differences between other results varied from a few to as much as 110 per cent.

Furthermore, it was not possible to obtain a reasonable corrected value by the use of the equation. When the results were corrected according to the equation, the values obtained were 11 to 1500 per cent too low, as shown in figure 2. Since the readings taken with the green filter do not follow Beer's law of proportionality when aqueous alkali is used, such a discrepancy would be expected. However, when the Holtorff-Koch method was applied to the corresponding ketonic fractions, the results were, within the limits of experimental error, identical with those obtained by the Callow procedure. This also is illustrated in figure 1. Therefore it appears that if the Holtorff-Koch technic is to be used as an accurate method of assay, the determination must be made on the ketonic fraction. When a relatively large

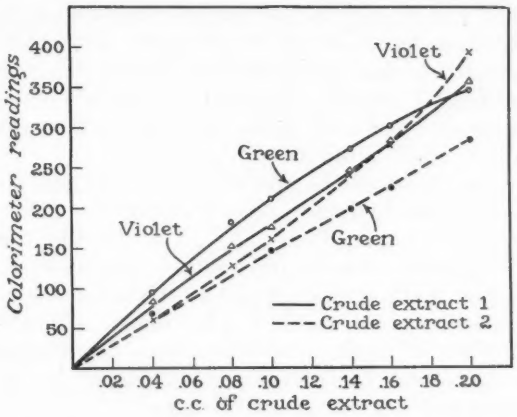



FIG. 5. Relation between amount of crude extract and colorimeter readings with green and violet filters, using the Holtorff-Koch procedure.

amount of 17-ketosteroid is present, the difference between the results obtained by the two technics is not serious but when the amount of 17-ketosteroid is small, the Holtorff-Koch method introduces a serious error.

In order to make certain that the deviation from proportionality for the crude extract observed with the Holtorff-Koch method (fig. 3) was not due to any variations in our technic the amount of color given by varying amounts of dehydro-iso-androsterone and ketonic fractions was determined. Strict proportionality between the amount of substance and color was obtained with dehydro-iso-androsterone up to at least 90 micrograms and with most of the ketonic fractions; a few ketonic fractions were found for which apparently there was a slight deviation from the linear relationship. The disagreement observed when the two procedures were applied to crude extracts, and the agreement when they were applied to ketonic fractions, are thus

explained. The data on the ketonic fractions are given in figure 4. It was further observed that in certain crude extracts the $V/G$ ratio gradually increased as the concentration increased when the Holtorff-Koch method was used. Two examples are given in figure 5. Any significant change in this ratio further invalidates the use of any correction equation.

## SUMMARY

In agreement with the work of Talbot, when the Callow method was employed for the determination of 17-ketosteroids in crude extracts of urine, the use of an equation for the correction of the colorimeter readings was found to give results which agreed closely with those obtained by assay of the ketonic fractions separated from the crude extracts. The validity of the color correction equation was more firmly established by recovery experiments in which crystalline dehydro-iso-androsterone was added to crude extracts and to non-ketonic fractions. Therefore, reasonably accurate results may be expected for the assay of crude extracts when the correction equation is used with the Callow absolute alcohol method.

The equation could not be applied to the readings obtained with the Holtorff-Koch method since the color given by the crude extract deviated significantly from Beer's law. A simple blank also did not furnish adequate correction for impurities in the crude extract. However, when either method was applied to the ketonic fraction, Beer's law was followed and both methods gave identical results. For these reasons, therefore, if accurate results are to be obtained with the Holtorff-Koch method the determination should be made on the ketonic fraction and not on the crude extract.

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# INHIBITING EFFECT OF ADRENOCORTICOTROPIC HORMONE ON THE GROWTH OF MALE RATS[1]

HERBERT M. EVANS, MIRIAM E. SIMPSON AND CHOH HAO LI
*From the Institute of Experimental Biology, University of California*
BERKELEY, CALIFORNIA

SOME years ago it was shown (Moon, 1937) that the partially purified adrenocorticotropic hormone retarded the somatic growth of castrated rats. A similar effect on normal rats was also reported by Ingle, Higgins and Kendall (1938) and Wells and Kendall (1940) with adrenal cortical hormones. Recently the antagonistic action of the adrenocorticotropic hormone to growth hormone in hypophysectomized rats has been clearly demonstrated (Marx, Simpson, Li and Evans, 1943). In this paper data are presented to show the growth inhibition of adrenocorticotropic hormone in normal and gonadectomized male rats. The pure hormone was used and was isolated from the sheep pituitaries by the method of Li, Simpson and Evans (1942).

Table 1 summarizes the results of the study of growth inhibition using both immature and adult male rats. The control group of 26-day-old rats gained twice as much as the experimental group. The food intake of these two groups was measured: the injected rats ate an

TABLE 1. EFFECT OF ADRENOCORTICOTROPIC HORMONE ON THE BODY WEIGHT OF NORMAL MALE RATS

| Age of Rats | Length of Treatment | Total Dose of Hormone | No. of Rats | Body Weight | | |
|---|---|---|---|---|---|---|
| | | | | Initial | Autopsy | Gain |
| *days* | *days* | *mg.* | | *gm.* | *gm.* | *gm.* |
| 26 | 30 | 30.0[1] | 12 | 65 | 145 | 80 |
| | 30 | 0.0 | 12 | 65 | 219 | 154 |
| 89 | 15 | 15.0[1] | 6 | 302 | 288 | −14 |
| | 15 | 0.0 | 6 | 301 | 336 | 35 |

[1] 1 mg. daily dose divided in 3 injections during 8-hour period; Saturday 2 injections, and Sunday, 1 injection only.

average of 12.0 gm. of food per day whereas the controls ate 12.8 gm. The inhibiting effect of the hormone on body weight gain cannot be accounted for by the difference in food consumption.

In adult male rats the hormone caused a reduction of body weight (table 1). Whereas the uninjected controls continued to gain weight in

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the 15-day period, some of the injected rats actually lost weight while others failed to gain.

Such growth inhibition was also observed in gonadectomized animals as shown in table 2. This effect did not occur in adrenalec-

TABLE 2. EFFECT OF ADRENOCORTICOTROPIC HORMONE ON THE BODY WEIGHT OF ADRENALECTOMIZED AND GONADECTOMIZED MALE RATS

| Type of Rats | Age of Rat, Experiment Began | Length of Experiment | Total Dose | No. of Rats | Body Weight | | |
|---|---|---|---|---|---|---|---|
| | | | | | Initial | Autopsy | Gain |
| | *days* | *days* | *mg.* | | *gm.* | *gm.* | *gm.* |
| Gonadec- | 25 | 30 | 60.0[1] | 5 | 57 | 164 | 107 |
| tomized | 25 | 30 | 0.0 | 5 | 61 | 194 | 133 |
| Adrenal- | 26 | 30 | 30.0[2] | 10 | 75 | 188 | 113 |
| ectomized[3] | 26 | 30 | 0.0 | 10 | 72 | 186 | 114 |

[1] A partially purified fraction of adrenocorticotropic hormone was used; 2 mg. dose in one injection daily, beginning the day of operation.

[2] 1 mg. daily dose, divided in 3 injections during 8-hour period; Saturday 2 injections and Sunday, 1 injection only. Injections begun the day of operation.

[3] All adrenalectomized rats were given 1% NaCl drinking water. The food consumption was measured and was found to be the same in both injected and control groups.

tomized rats maintained on 1% NaCl in the drinking water (table 2). If the inhibition of growth is due to the adrenal cortical hormone secretion of the hypertrophied adrenals, caused by the administration of adrenocorticotropic hormone, it is to be expected that this phenomenon should not be observed in animals after removal of the adrenals.

## SUMMARY

The body growth of normal and gonadectomized male rats is inhibited by the pure sheep adrenocorticotropic hormone. The inhibition does not occur in adrenalectomized rats.

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# STUDIES ON THE MAINTENANCE OF PREGNANCY IN THE WHITE RAT[1]

FREDERICK N. ZEINER

*From the Department of Zoology, Indiana University*

BLOOMINGTON, INDIANA

OVARIECTOMY performed before implantation of fertilized ova in the pregnant animal terminates gestation and this result is universal in the species studied. Although removal of the ovaries after implantation does not disturb the normal course of gestation in some species, notably the human, the horse, and the monkey, abortion or resorption invariably occurs following ovariectomy, in other species of which the rat is an example (Allen, Hisaw and Gardner, 1939; Reynolds, 1939).

It has been assumed that the placenta secretes estrone and progesterone in sufficient amounts to maintain the pregnant state in those forms in which castration is not followed by the termination of pregnancy. As an example, the placentae of rats have been shown to produce estrone and progesterone (Selye, Collip and Thomson, 1935; Haterius, 1936; Kirsch, 1938), but it has been concluded that they do not secrete enough of these substances to maintain pregnancy, because pregnancy is terminated approximately 48 hours following ovariectomy (Harris and Pfiffner, 1929; Nelson and Haterius, 1930; Johnson and Challans, 1930, 1932; Pencharz and Long, 1932; Hain, 1934; Allen, Hisaw and Gardner, 1939; Reynolds, 1939). Since the fetuses of the rat are aborted, even in the presence of detectable quantities of progesterone and estrone produced by the placenta, it was thought that perhaps factors other than the quantity of placental secretion might be involved.

In contrast to the reports in the literature, preliminary observations in this laboratory indicated that fetal life following ovariectomy of the pregnant rat may continue for periods considerably longer than 48 hours. The necessity for more extensive data concerning effects of ovariectomy during pregnancy became apparent and is the purpose of this study of the rat.

Although sham operations and unilateral ovariectomies do not disturb the course of pregnancy in the rat (Johnson and Challans, 1930, 1932; Pencharz and Long, 1932; Haterius, 1935), there is some indication that traumatic shock may have a deleterious effect on pregnancy. It was demonstrated by the experiments of Haterius and

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Kempner which were designed to test the effects of uterine distention (1939), that it was possible to maintain pregnancy following two-stage ovariectomy in rats. It was not possible, however, to do so if ovariectomy was completed in one operation. It appears that shock would be reduced by the use of a two-stage ovariectomy. Attempts have been made in this study to determine if shock is in part responsible for the results of ovariectomy in the pregnant rat by the use of techniques of ovariectomy designed to reduce shock, and by the use of adrenal cortical extract which has been shown to counteract the effects of trauma (Selye, Dosne, Bassett and Whittacker, 1940; Selye and Dosne, 1940; Weil, Rose and Browne, 1940).

The effects of small amounts of progesterone, estrogen,[2] and combinations of these on the maintenance of pregannncy following ovariectomy were tested, and the results have been evaluated in the light of the extensive control data obtained in the earlier work. It should be possible to detect the effects of small amounts of endocrine substances which act to maintain pregnancy by comparison with adequate control data and in this way to estimate the threshold of action of these substances. Daily injections begun at the time of ovariectomy, single injections given at the time of ovariectomy, and implantation of pellets of crystalline progesterone have been utilized in this study.

It has been reported that embryos are killed by pressure of the uterine walls following ovariectomy of the pregnant rat (Selye, Collip and Thomson, 1935; Haterius and Kempner, 1939). Although no experiments were performed to confirm this, incidental observations on the effects of uterine pressure were made.

## MATERIAL AND METHODS

Young adult albino rats ranging in age from 100 to 150 days were used. The majority of the animals were purchased from Sprague-Dawley Incorporated, Madison, Wisconsin, but those used in the later experiments were obtained from the Harlan Small Animal Industries, Cumberland, Indiana. No differences could be detected in the reactions of the two strains.

The males were put in the cages with the females for periods of from 4 to 10 hours. Pregnancy was timed from the middle of the period in which spermatozoa were found in the vaginal smear and thus the maximum error in dating the pregnancy does not exceed 5 hours in any case.

Semi-sterile precautions were exercised in all surgical procedures, and ether anesthesia was employed. The dorsal approach with a single cutaneous incision was used in performing ovariectomy. In excising the ovaries, a hemostat was clamped over the region of junction of oviduct and uterus in such a way as to shut off the ovarian blood supply. The ovarian capsule, oviduct and occasionally the very tip of the uterus were removed with the ovary to make sure that no ovarian material remained in the animal. A 'pre-

[2] The progesterone (Proluton) and estrogen, estradiol benzoate (Progynon-B) used in this investigation were supplied through the courtesy of Dr. Erwin Schwenk, Schering Corporation, Bloomfield, New Jersey. Adrenal cortical extract was furnished by the Wilson Laboratories, Chicago, Illinois.

liminary' operation was used in a large part of this work. The ovary was drawn out through a small slit in the body wall musculature leaving the ovarian blood supply and oviduct intact. The incision was then loosely drawn together with silk thread in such a way that the ovary was held between the skin and the body wall musculature, but retained all its normal functional relationships. It subsequently was necessary only to cut the sutures in the skin to expose the ovary which then could be removed very rapidly with a minimum of trauma.

Transplantation of ovarian material to the ear was made into a pocket between the layers of skin of the ear. This was done by making a small incision at the base of the ear and then separating the layers of skin with blunt forceps. From one-third to one-half of an ovary was placed in this pocket. The animals were kept in individual cages following any surgical treatment.

All dilutions of progesterone and estrogen were made with sesame oil. It was impossible to make pellets of progesterone with amounts as small as 1 or 2 mg. so that the desired amount of progesterone was mixed with 2 or 3 mg. of glucose to provide the necessary bulk.

Four criteria have been utilized in determining the duration of pregnancy following ovariectomy. *a*) The nature and amount of vaginal discharge were checked 4 to 5 times a day with observations extending from 9:00 A.M. to 11:00 P.M.; *b*) an attempt was made to detect the presence of embryos by palpation and to determine by palpations on successive days if growth of the palpable masses was occurring; *c*) weights were taken daily (or more often if the vaginal bleeding was profuse); and *d*) the observations of weight, vaginal discharge and palpation were then evaluated in the light of the condition of the uterus and its contents at autopsy. Each of these items contributes to the knowledge of the intra-uterine condition and the combined observations provide for a reasonably accurate estimate of the duration of embryonic life following ovariectomy. Moderate bleeding from the vagina, for example, may indicate the termination of gestation, or it may only indicate the abortion of one or two embryos. If there were palpable masses in the uterus shortly before vaginal bleeding was observed and no palpable masses were detected after the appearance of vaginal bleeding and if during the same period there was a noticeable drop in weight of the animal, it could be concluded that abortion had occurred. If palpable masses were still present and the animal continued to gain weight after vaginal bleeding was observed, it was indicated that gestation had continued at least for some of the embryos. These conclusions were checked by autopsies.

Two groups of figures are used to indicate the duration of pregnancy following ovariectomy in a series. If at the time of autopsy there were no living embryos present, the data on bleeding and weight loss were used to estimate the duration of pregnancy. Autopsy was performed in the majority of cases about 100 hours after castration and living fetuses were often present. There is, obviously, no way to determine how long gestation might have continued beyond the time of autopsy in these animals. The time of autopsy has been used, therefore, in order to include such animals in a calculation of the mean duration of pregnancy. The number of rats that carried living fetuses at the time of autopsy and the number of living fetuses are indicated in the tables. A plus sign has been added to the average duration to indicate that this figure would be larger if it were possible to determine how long gestation might have continued in rats which carried living embryos at autopsy. If

living embryos were definitely palpable at one hundred hours, autopsy was delayed.

It should be emphasized that all figures given in the tables represent estimates of the duration of embryonic life of any embryos following ovariectomy and not the time to the abortion of the uterine contents which may or may not have been living at the time of abortion. Since some of the reports in the literature of the time of abortion following ovariectomy are based on the appearance of blood in the vagina, the time following ovariectomy when any type of vaginal discharge was first noted is given. These data and the fact that there is often a small amount of blood in the vagina of a non-pregnant rat one or two days after ovariectomy demonstrate the unreliability of using the criterion of vaginal bleeding alone in determining the time of abortion.

## RESULTS AND DISCUSSION

*Results of different methods of removing the ovaries.* Preliminary observations indicated that all rats did not abort immediately after ovariectomy, but some carried viable embryos for considerable periods. Series 17 in table 1 gives the results of one-stage bilateral castration in 46 pregnant rats. The mean duration of pregnancy was 80+ hours and, therefore, 32 hours longer than the time generally reported. Much individual variation was noted, however; one animal was found to have a living fetus at autopsy, 162 hours after ovariectomy, and several others carried living embryos longer than 100 hours. A consideration of these data suggests that the ovaries of a rat are less essential during pregnancy than usually believed and the presence of other factors is suggested.

Although operative shock alone does not cause the interruption of pregnancy (Johnson and Challans, 1930, 1932; Pencharz and Long, 1932; Haterius, 1935; personal observations), ovariectomy introduces the factor of trauma as well as endocrine imbalance and the question arises whether abortion might result from the combination of these two effects. It has been demonstrated that the adrenal cortical principle is liberated in large quantities as a result of traumatic shock (Selye, Dosne, Bassett and Whittacker, 1940; Selye, 1940). The results of series 32, which will be mentioned in detail later, indicate that cortical extract is detrimental to the pregnant state following removal of the ovaries. Haterius and Kempner (1939) were unable to maintain pregnancy in rats following one-stage castration although maintenance was obtained following two-stage ovariectomy. These observations, the results in series 17, of this report, and the indication that an excess of adrenal cortical principle is detrimental to the pregnant state following ovariectomy, suggest that shock plays a rôle in the causation of abortion following ovariectomy. Several methods of removing the ovaries have been utilized in an attempt to reduce the effect of trauma and thus make it possible to get a clearer view of any rôle played by operative shock.

The results obtained by modifying the surgical procedure are set forth in table 1. Pregnancy was maintained longer than 48 hours in all cases. The duration of fetal life in series 19 and 20 was definitely longer than in series 17 which serves as a control. The most striking results are found in series 19 in which the preliminary operation was performed before coitus. Only one operation was performed during pregnancy in this group and possible trauma was reduced to a minimum in that single operation. The average duration of pregnancy after ovariectomy was more than 108 hours and one animal was found

TABLE 1. EFFECT OF SURGICAL METHODS UPON DURATION OF PREGNANCY FOLLOWING OVARIECTOMY

| Series | Treatment | No. of Rats | Operation | | Average Duration hr. | Rats with Live Embryos at Autopsy | No. of Living Embryos | Vaginal Discharge hr. |
|---|---|---|---|---|---|---|---|---|
| | | | Day[1] | Range | | | | |
| 12 | Bilateral ovariect. | 46 | 13.7 | 11.0–16.7 | 80.2+ | 6 | 15 | 45.6 |
| 19 | Prelim.[2] before coitus | 13 | 13.5 | 12.9–15.0 | 108.1+ | 4 | 8 | 75.1 |
| 20 | Prelim. during preg. | 28 | 13.5 | 12.0–15.0 | 93.0+ | 7 | 13 | 51.9 |
| 21 | L. prelim. and rt. ovariect. during preg. | 20 | 12.8 | 10.1–16.0 | 82.4+ | 3 | 3 | 50.0 |
| 25 | Rt. ovariect. before coitus. L. prelim. during pregnancy | 9 | 14.0 | 13.7–14.5 | 85.5+ | 1 | 1 | 62.2 |
| 40 | Rt. ovariect. and L. prelim. before coitus | 16 | 13.9 | 13.7–14.9 | 80.1+ | 4 | 4 | 39.0 |
| 41 | Rt. ovariect. and L. prelim. before coitus. L. ovary put in ear | 10 | 13.5 | 12.7–13.9 | 79.8 | | | 44.3 |
| 42 | Rt. ovary put in ear before coitus | 15 | 13.4 | 11.9–13.9 | 96.8 | | | 42.0 |
| 32 | Prelim. during preg. and cortical extract following operation | 5 | 13.5 | 13.4–13.5 | 56.8 | | | 41.4 |

[1] Average time in days after coitus when all ovarian tissue removed excepting transplantation to ear in series 41 and 42.
[2] Ovaries pulled out between the skin and the body wall musculature (in a preliminary operation) in such a way that the functional connections were retained. Bilateral unless otherwise stated.

to be carrying a living fetus 187 hours after ovariectomy. The results in series 20, in which the preliminary operation was performed about 2 days before ovariectomy, are also noteworthy in a similar fashion because it is indicated by that group that a reduction of operative shock at the time of castration prolongs the duration of pregnancy. Maintenance of embryos in series 21 (right ovariectomy and left preliminary operation 2 days before complete ovariectomy), series 25 (right ovariectomy before coitus and left preliminary operation 2 days before complete ovariectomy), and series 40 (right ovariectomy and left preliminary operation before coitus), however, was not significantly greater than in series 17 and attempts to reduce traumatic shock beyond this have been unsuccessful.

It appears from the procedure used that trauma should be least in series 40, but the results in maintenance of pregnancy were no better than in series 17. A comparison of the number of embryos per rat in series 40 with the number of embryos found in the other series throws some light on this discrepancy. It was found that the average number of implantations per rat in our colony, based on counts from

188 pregnant rats, was 4.43 in each uterine horn. The average number of implantations per uterine horn in cases where one or more living embryos were found at autopsy was 3.73. The average number of implantations per horn found in series 19 in which the best results were obtained was 1.96. These data indicate that better results were obtained in the maintenance of pregnancy if the original number of embryos was small. The concentration of decomposition products which result from the death of the fetuses will vary with the number of dead embryos in the uterus and the fate of the last surviving embryos of a litter might be expected to be partially determined by the local concentration of decomposition products.

It is interesting to note in the light of these observations that the average number of implantations in the single pregnant uterine horn of the rats in series 40 was 6.4. The concentration of decomposition products in the single uterine horn of the rats in this series would be much higher than in the case of a rat from series 19 and it is believed that the results obtained in series 40 can be explained in this way.

The technique of transplanting ovarian tissue to the ear was employed in order to test further the relative importance of shock and endocrine imbalance in causing abortion following ovariectomy. Nelson and Haterius (1930) have shown the feasibility of maintaining pregnancy following ovariectomy by means of ovarian grafts. If ovarian tissue transplanted to the ear could maintain pregnancy, it would be possible to remove that tissue with a minimum of shock by cutting off the ear and thus a good method of comparing the effects of shock and endocrine imbalance would be presented. The right ovary in series 41 was removed, and the left ovary was pulled out between the skin and the body wall before coitus. The left ovary was transplanted to the ear at the 14th day of pregnancy. Pregnancy was not maintained. Since the animals had irregular estrous cycles a week or two later, it was obvious that the transplants were functional. This suggested the possibility that the transplants in series 41 had not become vascularized soon enough to prevent abortion. Accordingly, in series 42, the right ovary was transplanted to the ear before coitus and the left ovary was then removed during pregnancy. Again pregnancy was not maintained. The grafts did not appear to 'take' satisfactorily presumably because of the influence of the normal left ovary. The grafts hypertrophied after the left ovary had been removed, but the level of secretion was probably too low at this time to maintain pregnancy. Later the animals again had regular cycles. It appears that if the technical difficulties of maintaining pregnancy by ovarian ear grafts could be overcome, much could be learned about endocrine and traumatic factors. Many of the animals with ovarian grafts had greatly prolonged estrous periods, and this suggests that ovarian function in the cooler environment of the ear is not entirely normal. Evidence has been presented, however, that a large reduction in the

amount of ovarian tissue will give rise to long periods of estrus alternating with long periods of diestrus (Lipschütz and Osnovikoff, 1932; Lipschütz, 1936a, b). The data in this study are inadequate to aid in the interpretation of the quantitative or thermal factors which may be acting.

Adrenal cortical extracts have been shown to counteract the effects of traumatic shock (Selye, Dosne, Bassett and Whittacker, 1940; Selye and Dosne, 1940; Weil, Rose and Browne, 1940). An attempt was made in series 32 to eliminate the shock factor involved in ovariectomy by the use of cortical extract. The animals were given 8 injections of 0.35 cc. each of adrenal cortical extract at 2-hour intervals following the operation. Although only 5 animals were used, the results were uniform. The extract had a marked detrimental effect because the average duration of pregnancy following castration was only 57 hours. Series 20 served as the uninjected control for the experiment. It is interesting that this result is contradictory to the demonstration of a progestational action of desoxycorticosterone acetate (Leathem and Crafts, 1940; Burdick and Konanz, 1941). Burdick and Konanz (1941) found that desoxycorticosterone acetate maintained both embryos and corpora lutea in pregnant mice, but they state that this substance may have a detrimental action on pregnancy when given in large quantities. Further evidence of possible detrimental action of cortical hormone was found by Tobin (1941). He removed both the ovaries and adrenals on the 12th day of pregnancy in rats and observed fetal growth for 2 to 8 days before absorption or abortion. Since pregnancy was maintained much longer in Tobin's experiments than was reported following castration alone, it is possible that the endogenous adrenal cortical principle may be responsible for the difference in results. This would correspond to the effects of the injection of adrenal cortical extract in series 32.

We may conclude that shock is a factor in hastening abortion following ovariectomy in the pregnant rat, but it has been impossible thus far to determine the relative importance of shock and endocrine imbalance in terminating pregnancy.

*Effect of small amounts of estrogen, progesterone, or both.* It was thought that, by the use of the extensive control data obtained in this work, it would be possible to detect the effect of small amounts of endocrine substances which act to maintain pregnancy, and in this way to estimate the threshold of action of these substances. Series 20 was selected to serve as a control for all work that involved the administration of estrogen, progesterone, or both. Estrogen was injected daily and progesterone was given either daily or in a single massive dose. The results of these experiments are summarized in table 2.

Neither estrogen, nor progesterone, nor combinations of these substances produced outstanding results in the amounts given. Pregnancy

was maintained somewhat longer with 10.0 I.U. of estrogen per day than with 3.3 I.U., but in neither case was the duration of pregnancy as great as in the controls. Pellets of crystalline progesterone produced only slightly better results than were obtained with progesterone in oil. A comparison of series 30 and 47 with series 35 indicates that better results were obtained with a massive dose of progesterone at the time of ovariectomy than with the same amount divided into daily injections. Two mg. of crystalline progesterone given at the time of castration prolonged pregnancy for a short time, but in series 50, with only 6 animals, the use of 3.0 mg. produced poorer results

TABLE 2. EFFECT OF SMALL AMOUNTS OF PROGESTERONE, ESTROGEN, OR COMBINATIONS OF THE TWO ON THE DURATION OF PREGNANCY FOLLOWING OVARIECTOMY

| Series | Injection Material[1] | Day | No. of Rats | Operation[2] | | Average Duration hr. | Rats with Live Embs. at Autopsy | No. of Living Embs. | Vaginal Discharge hr. |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Day | Range | | | | |
| 29 | 0.5 prog. | 13 | 6 | 14.0 | 13.7–14.2 | 80.3+ | 2 | 2 | 51.0 |
| 30 | 1.0 prog. | 13 | 18 | 13.8 | 13.6–14.2 | 86.4+ | 2 | 3 | 59.0 |
| 31 | 1.5 prog. | 13 | 8 | 14.2 | 13.9–14.7 | 92.8+ | 2 | 2 | 59.4 |
| 47 | 1.0 prog.[2] | 14 | 10 | 13.8 | 13.6–14.3 | 90.2+ | 3 | 3 | 76.0 |
| 48 | 1.0 prog.[2] | 13 | 4 | 13.8 | 13.7–13.9 | 83.7 | 0 | 0 | 64.0 |
| 49 | 2.0 prog.[2] | 14 | 12 | 14.2 | 13.7–14.4 | 103.1+ | 3 | 6 | 93.6 |
| 50 | 3.0 prog.[2] | 14 | 6 | 14.2 | 13.7–14.5 | 90.0+ | 1 | 2 | 61.3 |
| 59 | 3.3 est. | D | 6 | 14.0 | 13.7–14.8 | 73.0 | 0 | 0 | 47.6 |
| 60 | 2.0 prog.[2] 3.3 est. | 14 D | 10 | 13.7 | 13.7–13.8 | 96.3+ | 5 | 7 | 74.4 |
| 66 | 10.0 est. | D | 8 | 13.8 | 13.7–13.9 | 77.1+ | 2 | 4 | 64.5 |
| 76 | 2.0 prog.[2] 10.0 est. | 14 | 4 | 14.0 | 13.7–14.8 | 97.3+ | 3 | 10 | 90.0 |
| 33 | 0.1 prog. | D | 9 | 14.0 | 14.0–14.1 | 81.9+ | 1 | 1 | 51.5 |
| 35 | 0.25 prog. | D | 14 | 13.8 | 13.7–14.0 | 81.6+ | 6 | 8 | 40.0 |
| 20 | Control | | 28 | 13.5 | 12.0–15.0 | 93.0+ | 7 | 13 | 51.9 |

[1] Progesterone expressed in mg.; estrogen in I.U.
[2] Crystalline progesterone implanted in pellet form.
[3] Day of pregnancy when ovariectomized.
D Daily injection beginning at ovariectomy.

than were obtained with 2.0 mg. Three and .3 I.U. of estrone per day did not enhance the action of 2.0 mg. of progesterone given at the time of castration (series 60). If 10.0 I.U. per day of estrogen were given to animals which received 2.0 mg. of crystalline progesterone, pregnancy was maintained somewhat longer than in controls, and, since the percentage of viable embryos found at autopsy was higher than in any other series, series 76 is considered as having the best results. It must be pointed out, however, that only 4 rats were used in this series. Of these, 3 rats castrated at the 14th day of pregnancy carried viable embryos much longer than did one rat castrated at nearly the 15th day.

It is impossible at this time to explain the fact that in some cases the duration of pregnancy in the experimental series was shorter than in the controls. Although large quantities of estrone are known to disturb the normal course of pregnancy, there is no evidence that the small amounts used in this work have such an effect. It was suggested that the handling of the animals during daily injections might have a detrimental effect, but this seems without foundation since the animals were so tame that they struggled little if any while being injected. Although the small amount of progesterone utilized could not

be expected to maintain pregnancy until term in ovariectomized rats, one might expect to find a definite increase in the duration of fetal life with the amounts used, if progesterone is as active as is generally believed. The fact that relatively large quantities of progesterone must be used to maintain pregnancy in the castrated animal may indicate a very high degree of ovarian and probably placental production of progesterone. Nevertheless, since rather large quantities of progesterone had to be used before any effect at all could be noted in the current experiments, it is indicated that progesterone is relatively ineffective in the maintenance of pregnancy in the castrated rat.

*Observations on the ratio of the number of placentae to the number of embryos.* Because of the demonstration by Haterius (1935, 1936)

TABLE 3. PLACENTA:FETUS RATIO OF RATS WITH LIVING EMBRYOS AT AUTOPSY

| Uninjected Series | | | | Series Receiving Estrogen, Progesterone, or Both | | | |
|---|---|---|---|---|---|---|---|
| Ser. | No. of rats | No. of placentae | No. of embryos | Ser. | No. of rats | No. of placentae | No. of embryos |
| 17 | 4 | 13 | 7 | 29 | 2 | 3 | 2 |
| 19 | 5 | 13 | 9 | 30 | 2 | 4 | 3 |
| 20 | 7 | 30 | 13 | 31 | 2 | 5 | 2 |
| 21 | 4 | 8 | 4 | 33 | 1 | 5 | 1 |
| 25 | 2 | 2 | 2 | 35 | 6 | 13 | 8 |
| 40 | 4 | 6 | 4 | 47 | 3 | 6 | 3 |
| | | | | 48 | 0 | 0 | 0 |
| | | | | 49 | 3 | 7 | 6 |
| | | | | 50 | 2 | 8 | 3 |
| | | | | 59 | 0 | 0 | 0 |
| | | | | 60 | 5 | 12 | 7 |
| | | | | 66 | 2 | 4 | 4 |
| | | | | 76 | 3 | 11 | 10 |
| Total | 26 | 72 | 39 | | 31 | 78 | 49 |
| Total ratio | | 1.84 ± .039:1 | | | | 1.59 ± .214:1 | |
| Embryos per rat | | 1.50 ± .169 | | | | 1.58 ± .190 | |

that pregnancy is maintained in the castrated rat by a high placenta:fetus ratio, all rats with living embryos at autopsy were examined for the presence of extra living placentae. It was thought that a fortuitous high placenta:fetus ratio was a result of abortion of embryos with retention of living placentae might account for those cases in which living embryos were found at times considerably beyond the 48 hour limit given in the literature. The results of these observations are listed in table 3.

The average placenta:fetus ratio for the uninjected series is 1.84 ± .039:1 and it is 1.59 ± .214:1 in the injected series. The highest ratio found, 5:1, occurred in only one instance and in many rats the ratio was 1:1. This is in contrast to the results of Haterius (1936) who found that pregnancy was not maintained without extra placentae. We might expect more embryos to be maintained per rat in

the injected series than in the uninjected series even though the duration of pregnancy was not increased, but there was no outstanding difference (table 3). Consequently the small difference in the ratios is not explained by assuming an endocrine function of the extra placentae. It may be concluded, therefore, that the presence of extra placentae was not responsible for prolonged gestation following castration in these experiments.

Another possibility has been considered as a factor in maintenance of the embryos, namely, that the prolonged gestation may be a result of the action of the dead fetuses and placentae in distending the uterus comparable to the distention produced by wax in the experiment of Haterius and Kempner (1939). It would appear, however, that the products of decomposition of the embryos would more than offset any beneficial action of distention.

*Observations on the effect of uterine distention.* Evidence has been presented that embryos are killed by pressure following ovariectomy (Selye, Collip and Thomson, 1935; Haterius, 1936; Haterius and Kempner, 1939). Although no experiments were performed specifically to test this, incidental observations tend to support it. Rats were found occasionally which had a single implantation in one horn of the uterus. When a living embryo was present at autopsy in such cases the uterus was much constricted on either side of the embryo, and often the embryo showed effects of pressure inasmuch as the head was cone-shaped and usually had a blood sinus at the apex of the cone. This condition was never found if the uterus was distended at the anterior end of the embryo by other embryos or their remains.

## SUMMARY

A study of the effect of ovariectomy on pregnant rats indicates that pregnancy may continue for much longer periods following ovariectomy than the 48 hours generally reported. The average duration of pregnancy following bilateral ovariectomy of 46 animals was 80 hours. One animal was carrying a living fetus at autopsy 162 hours after ovariectomy.

The duration of pregnancy following ovariectomy is prolonged by the improvement of operative techniques, such as two-stage ovariectomy, which reduces the amount of operative shock. Best results have been obtained by combining a two-stage procedure with the technique of pulling the ovaries out between the skin and the body-wall musculature where they may be reached later with a minimum of delay and trauma. Using these methods the average duration of pregnancy in one series was greater than 108 hours. This is 28 hours longer than in the control series. Pregnancy continued for more than 187 hours in one case. Although it has not been possible thus far to determine the extent of the rôle of shock by its complete elimination from the picture, it is concluded that trauma is a definite factor in hastening abortion.

It was thought that the use of ovarian ear grafts would provide a way of removing the source of ovarian hormone with an absolute minimum of trauma. However, in the limited amount of work done, no success in maintaining pregnancy with ovarian ear grafts was attained so that this method of studying the effects of trauma was unsuccessful.

The effects of small amounts of estrogen, progesterone and combinations of these on the duration of pregnancy following ovariectomy were tested and the results compared with the extensive control data obtained in the previous work. No definite maintenance effects were noted until quantities of 2.0 mg. of crystalline progesterone at the time of castration followed by 10.0 I.U. per day of estrogen were given.

The increased duration of pregnancy found in these experiments was not due to an excess of placentae which might result in a high placenta : fetus ratio.

It was observed that embryos were probably killed by pressure of the uterine wall following removal of the ovaries in some instances.

The author wishes to express his appreciation for the many helpful suggestions and for the constant encouragement of Dr. W. R. Breneman under whose direction this study has been undertaken.

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# RELATION OF CERTAIN ENDOCRINE GLANDS TO BODY WEIGHT IN GROWING AND MATURE NEW ZEALAND WHITE RABBITS[1,2]

H. H. KIBLER, A. J. BERGMAN AND C. W. TURNER
*From the Department of Dairy Husbandry, University of Missouri*
COLUMBIA, MISSOURI

IN the course of a series of experiments in this laboratory during the past few years, a considerable number of normal New Zealand White rabbits have served as controls for experiments. When these animals were killed, the pituitary, thyroid, parathyroid and adrenal glands and the ovaries and testes were weighed. Kibler, Bergman and Turner (1942) have reported a study of the relation of the pituitary weight to body weight in these animals, while Campbell and Turner (1942) similarly studied the parathyroid glands. The present paper reports the relation of body weight to thyroid, adrenals, ovaries and testes weights of these same animals.

It has been pointed out that endocrine gland weights do not usually vary directly with body weight, but to some power of body weight (for references see Mixner, Bergman and Turner, 1943). The relative growth equation $Y = aX^b$ has been used widely in studies relating organ or gland weight to body weight (Brody and Kibler, 1941).

The determination of the relation between body weight and endocrine gland weight of the common laboratory animals is believed to be of value not only as a check on the normality of small groups of control animals, but also as a means of roughly approximating the dosage of hormones which act upon these glands, in relation to body weight.

## EXPERIMENTAL PROCEDURE

The New Zealand White rabbits included in this study were fed a ration of mixed grain and alfalfa hay, kept at an environmental temperature between 50 and 70° F., and killed during the fall, winter and early spring months. Neither the males nor the females weighing less than 3000 gm. were used for breeding purposes. The animals were killed by injecting air into an ear vein. Body weights were recorded to the nearest gram and the thyroid, the paired adrenals and gonads were weighed to the nearest 0.1 mg.

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[2] The authors are indebted for a portion of these data to Messrs. Skat Holst and P. T. Cupps for use of unpublished data on the endocrine gland and body weights of individual rabbits.

TABLE 1. BODY WEIGHT AND GLAND WEIGHT IN GROWING AND MATURE NEW ZEALAND WHITE RABBITS

| Number of Animals | Body Weight Group gm. | Average Weight | | | |
|---|---|---|---|---|---|
| | | Body gm. | Thyroid mg. | Adrenals mg. | Gonads mg. |
| | | *Males* | | | |
| 9 | 51– 150 | 124 | 12.1 | 13.8 | 18.9 |
| 1 | 151– 250 | 210 | 23.9 | 16.3 | 26.1 |
| 4 | 251– 350 | 325 | 27.4 | 36.0 | 59.7 |
| 11 | 351– 450 | 428 | 33.8 | 45.3 | 82.1 |
| 25 | 451– 550 | 493 | 34.8 | 43.0 | 89.4 |
| 18 | 551– 650 | 606 | 35.7 | 51.7 | 117 |
| 6 | 651– 750 | 700 | 38.6 | 62.7 | 148 |
| 11 | 751– 850 | 808 | 58.6 | 57.0 | 148 |
| 15 | 851– 950 | 934 | 75.5 | 79.2 | 226 |
| 32 | 951–1100 | 1023 | 76.2 | 77.9 | 284 |
| 12 | 1101–1300 | 1199 | 83.5 | 104 | 399 |
| 26 | 1301–1500 | 1452 | 95.6 | 136 | 1112 |
| 25 | 1501–1700 | 1586 | 102 | 124 | 1221 |
| 21 | 1701–1900 | 1813 | 114 | 163 | 1875 |
| 29 | 1901–2250 | 2043 | 139 | 177 | 2639 |
| 32 | 2251–2750 | 2513 | 152 | 214 | 4197 |
| 32 | 2751–3250 | 3003 | 170 | 301 | 6045 |
| 5 | 3251–3750 | 3587 | 240 | 554 | 8014 |
| 4 | 3751–4250 | 4028 | 245 | 633 | 9760 |
| 2 | 4251–4750 | 4300 | 260 | 634 | 7420 |
| 320 | | | | | |
| | | *Females* | | | |
| 5 | 51– 150 | 119 | 13.1 | 13.6 | 15.0 |
| 1 | 151– 250 | 248 | 18.7 | 27.7 | 25.7 |
| 6 | 251– 350 | 311 | 23.0 | 39.1 | 18.1 |
| 10 | 351– 450 | 411 | 26.5 | 41.5 | 17.8 |
| 42 | 451– 550 | 512 | 37.5 | 47.5 | 20.5 |
| 24 | 551– 650 | 588 | 39.5 | 46.7 | 21.1 |
| 7 | 651– 750 | 677 | 46.3 | 54.2 | 24.6 |
| 2 | 751– 850 | 760 | 30.8 | 54.6 | 23.5 |
| 9 | 851– 950 | 910 | 56.0 | 76.1 | 34.0 |
| 28 | 951–1100 | 1013 | 77.3 | 83.1 | 36.4 |
| 22 | 1101–1300 | 1195 | 86.9 | 105 | 54.7 |
| 18 | 1301–1500 | 1452 | 120 | 144 | 110 |
| 22 | 1501–1700 | 1568 | 112 | 152 | 136 |
| 16 | 1701–1900 | 1795 | 115 | 167 | 134 |
| 26 | 1901–2250 | 2067 | 136 | 195 | 166 |
| 32 | 2251–2750 | 2556 | 158 | 267 | 257 |
| 29 | 2751–3250 | 3045 | 177 | 383 | 364 |
| 27 | 3251–3750 | 3491 | 202 | 494 | 546 |
| 24 | 3751–4250 | 3989 | 215 | 531 | 712 |
| 12 | 4251–4750 | 4504 | 207 | 503 | 900 |
| 362 | | | | | |

These data presented in table 1 were classified on the basis of body weight for the two sexes. The class interval was 100 gm. up to 950 gm., 200 gm. up to 1900 gm. and 500 gm. for the remainder of the groups.

## ANALYSIS OF DATA

*Thyroid and body weight.* When plotted on logarithmic paper, the individual observations on thyroid and live body weights for 320 male and 362 female rabbits fall along a straight line (fig. 1). As the

sexes did not appear to differ significantly, a single equation of the form $\log Y = a + b \log X$ was fitted to the data by the method of least squares. When transformed to the usual form, the equation became:

(1) male and female $Y$ (thyroid weight) $= 0.156X^{0.88}$ (body weight)

As exponent $b$ is less than 1.0, the ratio of thyroid weight to body weight decreases as body weight increases.

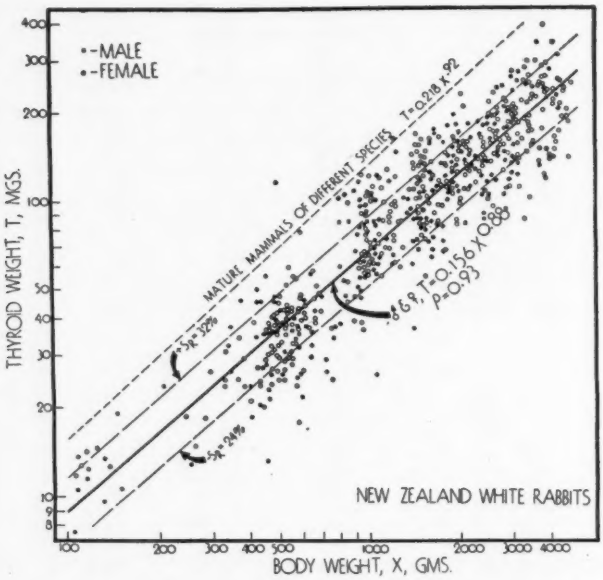


FIG. 1. Relation of thyroid weight to body weight in growing and mature New Zealand White rabbits; plotted on a logarithmic grid. The broken lines on both sides of the equation line represent the standard error of estimate, $S_r$, including between them two-thirds of the data; the letter rho represents the index of correlation. The dotted line representing mature mammals of different species is taken from the paper by Brody and Kibler (1941).

It is interesting to compare the value of the exponent $b$ determined for the rabbit with other similar data (table 2).

With the exception of the horse and man, the ratio of thyroid weight to body weight decreases as body weight increases. In this connection it is interesting to note that the relation of energy metabolism to body weight in mature horses has been found to be directly proportional, *i.e.*, with an exponent $b$ of 1.01 in comparison to 0.73 for all species (Kibler and Brody, 1943).

*Adrenal and body weight.* The adrenal weights of the two sexes also appeared sufficiently homogenous to plot together. However, it was noted that there was a change in the slope of the line at the 2500 gm.

TABLE 2. VALUE OF THE EXPONENT *b* FOR THYROID AND BODY WEIGHT IN VARIOUS SPECIES

| Species | Sex | b Exponent |
|---|---|---|
| Rats[1] | | 0.81 |
| Fetal and new born cats[1] | | 0.77 |
| Premature and new born horses[1] | | 0.95 |
| Horses over 1 year[1] | | 1.17 |
| Mature animals of different species[1] | | 0.92 |
| Guinea pig[2] | male | 0.73 |
| Guinea pig[2] | female | 0.83 |
| Man[3] | male & female | 1.08 |

[1] Brody and Kibler, 1941.
[2] Mixner, Bergman and Turner, 1943.
[3] Brody (in press).

body weight level (fig. 2). An equation was fitted to the adrenal weights for 261 male and 250 females with body weight under 2500 gm.

(2) male and female $Y$ (adrenal weight) $= 0.128X^{0.94}$ (body weight) (under 2500 gm. body weight)

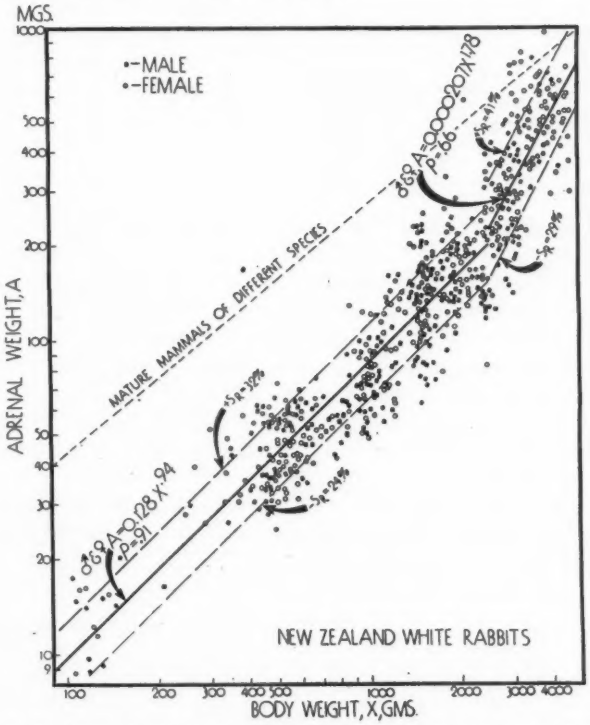


FIG. 2. Relation of adrenal weight to body weight in growing and mature New Zealand White rabbits. There is a marked rise in the rate of increase of adrenal weight with body weight in animals weighing over 2500 gm. The dotted line representing mature mammals of different species is from Brody and Kibler (1941).

In this equation the $b$ value of 0.94 indicates that the rate of adrenal development closely follows that of body weight up to 2500 gm.

The data for body weights over 2500 gm. include 59 males and 111 females. The regression equation for these data is presented in figure 2.

(3) Male and female $Y$ (adrenal weight) $= 0.000207X^{1.78}$ (over 2500 gm. body weight)

As the exponent $b$ in equation 3 is more than 1, *i.e.* 1.78, it means that the adrenals of the rabbit weighing more than 2500 gm., increase in weight at a greater rate than does body weight.

The value of the exponent $b$ for other species is given in table 3.

TABLE 3. VALUE OF THE EXPONENT $b$ FOR ADRENAL AND BODY WEIGHT IN VARIOUS SPECIES

| Species | Sex | b Exponent |
|---|---|---|
| Immature dogs[1] | | 0.78 |
| Growing rats[1] | male | 0.77 |
| Growing rats | female | 0.77 |
| Fetal & new born cats | | 0.64 |
| Growing chickens | | 0.84 |
| Growing horses | | 0.71 |
| Mature mammals of different species[1] | | 0.80 |
| Guinea pig (under 500 gm.)[2] | male & female | 0.95 |
| Guinea pig (over 500 gm.)[2] | male & female | 1.79 |

[1] Brody and Kibler, 1941.
[2] Mixner, Bergman and Turner, 1943.

It will be seen that in the species studied, the ratio of adrena weight to body weight is of about the same order, the exponent $b$ of immature animals being less than 1 in all cases. In the guinea pig it has been observed, however, that in the body weight classes over 500 gm. the exponent $b$ is almost twice that of immature animals. The present data for the rabbit indicate a similar change in the ratio of adrenal weight to body weight above 2500 gm. It would seem reasonable to expect that the secretion of the adrenotropic hormone of the pituitary and the adrenal cortical hormones would increase following the break in the curve.

*Gonad and body weight, male.* In the study of the relation of the gonad weight to body weight, animals weighing under 800 gm. were excluded (fig. 3). An equation was fitted to the 243 individual data points for testis weight/body weight as follows:

(4) Male $Y$ (testis weight) $= 0.00000081X^{2.84}$ (body weight)

The $b$ value of the equation, 2.84 indicates that testis weight (above 800 gm. body weight) increases at a much greater rate than does body weight. *Female.* An equation was fitted to the 265 individual data points for the ovary weight/body weight as follows:

(5) Female $Y$ (ovary weight) $= 0.000027X^{2.04}$ (body weight)

The $b$ value of the equation 2.04 indicates that the rabbit ovary (above 800 gm. body weight) also increases at a much greater rate than does body weight.

Only limited data on the value of the exponent $b$ for other species are available. Mixner, Bergman and Turner (1943) reported values

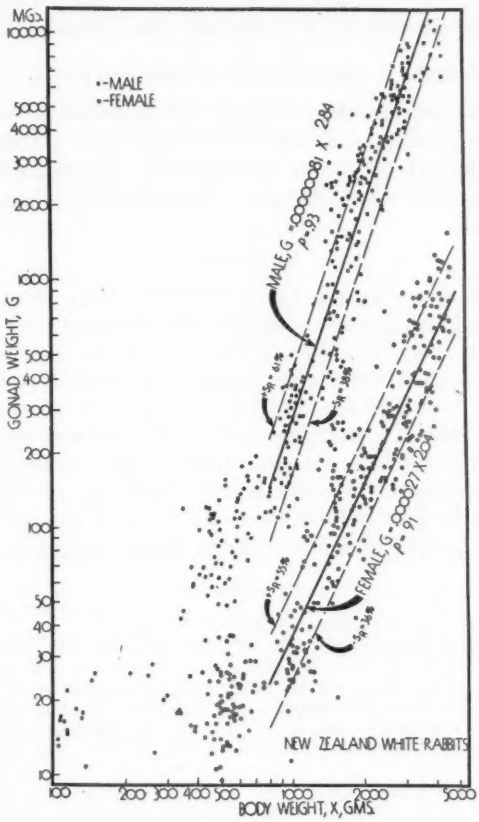


FIG. 3. Relation of gonad weight to body weight in growing and mature New Zealand White rabbits; plotted on a logarithmic grid. The testis increases in weight at a much greater rate with increasing body weight than any other endocrine gland.

of 2.32 for the gonads of the male guinea pig and of 0.76 for the female guinea pig. The $b$ values for the testes of the two species appear comparable, but the rabbit ovary increases in weight at a much faster rate than does the guinea pig. This may be due in part to the fact that the follicles of the rabbit ovary remain enlarged whereas the guinea pig ovary undergoes marked changes during the estrous cycle.

*Other endocrine glands.* For the sake of comparison, the equation of

the pituitary weight and body weight of these same rabbits determined by Kibler, *et al.* (1942), are included:

(6) Male $Y$ (pituitary weight) $= 0.265X^{0.58}$ body weight

(7) Female $Y$ (pituitary weight) $= 0.147X^{0.68}$ body weight

The comparable equation on the weights of the parathyroid glands of these rabbits presented by Campbell and Turner (1942) follow:

(8) Male and female $Y$ (parathyroid weight) $= 5.541X^{0.72}$ body weight

Of the endocrine glands studied, it will be noted that the pituitary has the lowest exponent $b$. The other endocrine glands in order of increasing exponent $b$ follow: parathyroid, thyroid, adrenal (under 2500 gm.), adrenal (over 2500 gm.), ovary (over 800 gm.) and testis (over 800 gm.).

## SUMMARY

The relative growth equation $Y = aX^b$ has been used to relate the thyroid, adrenal and gonad weights of 320 male and 362 female growing and mature New Zealand White rabbits to their body weight. The equations were fitted to the data by the method of least squares. The exponents $b$ of 0.88 for the thyroid and 0.94 for the adrenals of animals under 2500 gm. indicate that the ratio of the weights of these glands to body weight decreases as body weight increases. The exponent $b$ for the adrenals of animals over 2500 gm. of 1.78, ovaries of 2.04 and testes of 2.84 indicate that the ratio of the weights of these glands to body weight increases as body weight increases.

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# NOTES AND COMMENTS

## RECOVERY OF INJECTED CHORIONIC GONADOTROPIN FROM RABBIT URINE[1]

THE present study was undertaken to determine how much of injected chorionic gonadotropin is excreted in the urine of rabbits. These data appear pertinent to a comprehensive understanding of the biologic action of gonadotropins.

Stamler (1937), working with dogs, reported the recovery from urine of 11.2 per cent of injected chorionic gonadotropin in "over 20 hours" following the administration of the hormone. In a study conducted on 10 rats, Zondek and Sklow (1940) reported the recovery from the urine of 1 per cent of injected chorionic gonadotropin at the end of 4 hours and of 2 and 5 per cent at the end of 20 hours.

*Methods and materials.* Six rabbits (previously isolated for 21 days) weighing 2 to 2.5 kg. were each injected intravenously with chorionic gonadotropin[2] in a dose of 600 I.U The rabbits were kept in specially constructed metabolism cages equipped with wire-screened floors permitting the urine to drop onto galvanized iron funnels emptying into collection bottles. Fecal matter was retained on the floor screening and removed daily. The receivers contained chloroform (5 cc. per 100 cc. of urine) as a preservative. Specimens were placed in a refrigerator with a minimum delay following voiding.

*Technic of gonadotropin extraction.* The urine was extracted for gonadotropin as follows:

1. Urine was precipitated with 4 volumes of 95 per cent alcohol and allowed to settle overnight in refrigerator.
2. Following decantation of the supernatant the following morning, the precipitate was treated as follows:
   a. Washed 3 times with a half and half mixture of 95 per cent alcohol and U.S.P. acetone using 200, 150 and 75 cc. volumes, respectively.
   b. Washed once with 100 cc. of acetone.
   c. Finally washed twice with 100 and with 50 cc. volumes of U.S.P. ether.
3. Following decantation of the final volume of ether, the precipitate was set up to dry under vacuum. Drying was completed usually in from 30 to 60 minutes.
4. The dried precipitate then was ground to a fine powder with mortar and pestle and stored in refrigerator until used.

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[1] Part of the expenses of these studies was defrayed by grants to one of us (E.C.H.) by the Research Council of Duke University and by Ayerst, McKenna and Harrison, Montreal, Canada.

[2] APL, supplied by Ayerst, McKenna and Harrison, Montreal, Canada.

Daily 24-hour precipitates were pooled in order that the resulting mixture represented the total 24-hour output of the 6 rabbits.

The total combined powder for each 24-hour period was taken to represent 100 per cent and aliquots were weighed representing 25 per cent, 12.5 per cent, 6.25 per cent, 4.7 per cent, 3.1 per cent, 1.6 per cent, 0.8 per cent, 0.4 per cent, 0.2 per cent and 0.1 per cent.

*Method of bio-assay.* Eight rats were used for each dosage level. Aliquots were extracted with sufficient tap water to allow for the injection of a total of 3 cc. representing each titer. The extracts were injected subcutaneously over a period of 3 days in 6 divided doses into immature female rats (Vanderbilt strain). The rats ranged in age from 21 to 25 days and in weight from 32 to 42 grams. The animals were killed 96 hours following the initial injection. The uteri were excised, emptied of fluid by pressing lightly between sheets of filter paper and promptly weighed on an analytical balance. A total of 426 rats was used in this study.

A minimum increase of 100 per cent in uterine weight over that of control rats injected with known negative urines was used as the end point of assay. Since increase in uterine weight was used as the bio-assay end point it was necessary to make certain that the gonadotropin extracts did not contain estrogens. Accordingly an amount of extract representing a 3.2 per cent aliquot of the 48-hour period was injected into a series of 8 spayed immature female rats, in a manner similar to the routine assay for gonadotropin as described above. At the end of 96 hours the uteri of the spayed animals showed no increase in weight in contrast to those of the intact immature female rats which showed an average increase in uterine weight of more than 500 per cent over that of normal uninjected controls.

*Relation of rat unit to international unit.* In order to determine the relation of our rat unit to the international unit the chorionic gonadotropin extract was assayed in our own Vanderbilt strain of rats. Dilutions representing 0.5, 1.0, 1.5, 2, 3 and 4 I.U. of the chorionic gonadotropin extract were injected into 8 rats and the uteri were weighed at the end of 96 hours. A rat unit was considered as that amount producing an average of 100 per cent increase in uterine weight at the end of 96 hours. According to this assay, the I.U. of the chorionic gonadotropin extract was found to be equivalent to 2 of our uterine weight units.

*Results.* The amount of gonadotropin recovered from the urine of the 6 rabbits each of which had been injected with 600 I.U. of chorionic gonadotropin is shown in table 1.

TABLE 1. GONADOTROPIN RECOVERED FROM POOLED URINE OF SIX RABBITS EACH OF WHICH WAS INJECTED WITH 600 I.U. OF CHORIONIC GONADOTROPIN

| Time after Injection of Gonadotropin | Amount of Gonadotropin Recovered | Percentage of Injected Hormone Recovered |
|---|---|---|
| *hours* | I.U. | |
| 24 | 125 | 3.5 |
| 48 | 63 | 1.7 |
| 72 | 11 | 0.3 |
| 96 | 8 | 0.2 |
| 120 | 0 | 0.0 |
| Total | 206 | 5.7 |

It will be noted that using the uterine weight as the bio-assay unit the total amount of gonadotropin recovered during the 96 hours after the injection was 206 I.U. which is equivalent to 5.7 per cent of the total amount of gonadotropin injected into the 6 rabbits.

*Summary and conclusions.* This study was undertaken to determine how much of injected chorionic gonadotropin was recoverable from the urine of rabbits. Each of 6 rabbits was injected intravenously with 600 I.U. of chorionic gonadotropin. The amount of gonadotropin recovered during the first 96 hours following the administration of the chorionic hormone was 5.7 per cent of the total amount injected. No gonadotropin was found in the urine after 96 hours.

A. A. SALMON
E. C. HAMBLEN

*From the Endocrine Division of the Department of Obstetrics and Gynecology, Duke University School of Medicine and Duke Hospital, Durham, North Carolina*

STAMLER, C. N.: *Bull. Biol. Med.* exp. USSR, III: 1. 35. 1937, quoted by Zondek, Sulman and Sklow, 1940.
ZONDEK, B., F. SULMAN AND J. SKLOW: *J. Endocrinol.* 2: 362. 1940.

# NEW BOOKS

THE INTERACTION OF DRUGS AND CELL CATALYSTS. Frederick Bernheim, Associate Professor of Physiology and Pharmacology, Duke University School of Medicine, Durham, North Carolina. Offset lithography. $2.25. ii and 86. Paper binding. Burgess Publishing Co., Minneapolis, Minn. Copyright 1942; second printing, 1943.

FEW persons, more's the pity, have sufficient energy or daring to write books like this. There is too much danger that a neck so far protruded may encounter some routine, oscillatory axe. The author, however, in courageous recognition of the temporary nature of hypothesis has thoughtfully printed his work by offset lithography and covered it with paper so that its ultimate disposal may be accomplished without a qualm.

Let us not carp then because we do not encounter a classic. The intention is not to enunciate but to suggest. In effect the author says, "We have not gotten very far in explaining the action of drugs by talking with Straub about concentrations inside and outside of cells, or about polarization of membranes, abstract receptors, rectangular hyperbolas, or adsorption isotherms; let us see what can be gotten together from the point of view of known enzyme systems." Half the monograph is therefore devoted to an attempt to correlate the biological effects of various drugs, with their influence on enzyme systems *in vitro*. The other half deals with enzyme systems involved in the destruction or detoxification of foreign substances. Although the underlying notions are not entirely new, the author appears to have performed a valuable though thankless task in assembling widely scattered data and asking the scientific world to consider them from his point of view. No attempt is made at exhaustive analysis, elaborate reconciliation of conflicting experiments, or logical synthesis. As the author wisely recognizes, the time is not ripe.

The net impression made upon this reviewer is that the chemist in his exploration of enzyme systems is fashioning powerful instruments for the dissection of biological processes. If he is but ten years beyond his training years the physiologist or pharmacologist wishing to employ these tools will need plenty of help in learning their possibilities and modes of manipulation. Such books as this, incomplete as they must be, are going to come in very handy.

R. S. MORISON